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THE DESIGN OF CYTOTOXIC-AGENT-ANTIBODY CONJUGATES

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ABBREVIATIONS

AFP = Alpha-fetoprotein
ALL = Acute lymphocytic leukemia
ARA-C = Cytosine-1-beta-D-arabinoside
BSA = Bovine serum albumin
CALLA = Common acute lymphoblastic leukemia antigen
CEA = Carcinoembryonic antigen
CLL = Chronic lymphocytic leukemia
CPK = Creatine kinase
DHFR = Dihydrofolate reductase
DTT = Dithiothreitol
ECDI = 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide
EGF = Epidermal growth factor
FUR = 5′-Fluorouridine
hCG = Human chorionic gonadotropin
hPL = Placental lactogen (chorionic somatomammotropin)
HSA = Human serum albumin
HTLV = Human T-cell leukemia virus
I_{50} = Concentration giving 50% inhibition
Ig = Immunoglobulin
LH = Luteinizing hormone
MAA = Melanoma-associated antigen
MAB = Monoclonal antibody
MTX = Methotrexate
NHS = *N*-hydroxysuccinimide
PAGE = Polyacrylamide gel electrophoresis
PAP = Prostatic acid phosphatase
PBS = 0.01 *M* Sodium phosphate, containing 0.145 *M* sodium chloride

PDT = Pyridyldithio
pGA = Polyglutamic acid
PP5 = Placental protein 5
SDS = Sodium dodecylsulfate
SMBE = *N*-succinimidyl m-(*N*-maleimido)-benzoate
SMBU = *N*-succinimidyl m-(*N*-maleimido)-butyrate
SPDP = *N*-succinimidyl-3-(2-pyridyldithio)-propionate
SPI = Pregnancy-specific glycoprotein
TAA = Tumor-associated antigen
TATA = Tumor-associated transplantation antigen
TLC = Thin-layer chromatography

I. INTRODUCTION

In spite of remarkable advances in cancer chemotherapy, most chemotherapeutic agents and ionizing radiations currently in use have a low therapeutic index,[1] i.e., they damage all proliferating cells without discriminating between neoplastic and normal tissues, including such vital tissues as bone marrow, lymphoid tissue, gastrointestinal and genitourinary epithelium, etc.[2] The search for methods of rendering agents tumor specific has led to the experimental use of a wide array of carriers with various degrees of tumor-specificity.[3,4] Nonspecific carriers include cells, liposomes, and polymeric drug formations.[3,4]

If cancer cells have distinctive molecules on their surface and they are accessible, then one can use specific ligands to carry cytotoxic agents selectively to the target tumor, e.g., hormones,[5-7] transferrin,[8] lipoproteins,[9] lectins,[10,11] and antibodies.[12] The availability of MAB against a wide variety of TAA has now led to the accumulation of a substantial body of evidence in support of antibody-mediated drug targeting. Ghose and Blair evaluated the status and prospects for treatment of cancer in 1978[12] and later updated this, emphasizing their own experience in the field.[13,14] They have outlined methods that are useful in evaluating the antitumor activity of drug-antibody conjugates in vitro and in clinically relevant in vivo tumor models.[13] They have also reviewed the evidence for the localization of both polyclonal and monoclonal anti-TAA antibodies in target tumors in vivo and the feasibility of using labeled antibodies for the detection and possible therapy of cancer.[15-17] The pioneering studies of Landsteiner on the linkage of small haptenic moieties to carrier proteins and the wide application of this approach in the production of antibodies for radioimmunoassay have made available novel and milder methods of linkage of chemotherapeutic agents and toxins to Ig. Ghose and co-workers have outlined the strategy of linkage and have described those methods that have been used or are potentially useful for the production of biologically active Ig-linked anticancer agents.[13,14,18]

This review will be confined to antibody-mediated drug and toxin delivery systems as applied to cancer therapy. The evidence for and against the basic tenets of this approach will be evaluated. That is, are there antigenic moieties on tumor cells that can be therapeutically exploited? Do antibodies against these antigens selectively localize in target tumors in vivo? Can antibodies transport an active cytotoxic agent to the site of action of the agent, especially when such sites are intracellular? Liposomes will be dealt with only when they have been coated with an anti-TAA antibody to target packaged drugs. The widespread (but still experimental) use of antibody-linked radionuclides for tumor detection will not be considered. A compilation of selected abstracts on tumor localization of radiolabeled antibodies is available.[19]

II. TUMOR MARKERS AND TUMOR-ASSOCIATED ANTIGENS AS TARGETS FOR ANTIBODY-MEDIATED DELIVERY OF CYTOTOXIC AGENTS

There is evidence that chemically or virus-induced experimental tumors may acquire antigenic moieties that are not demonstrable in normal homologous tissues.[20,21] TATA induced by chemicals such as methylcholanthrene are highly immunogenic, stable, and heritable in a given tumor line. However, an important feature is their polymorphism.[22] For example, each methylcholanthrene-induced tumor appears to express a distinctive TATA, even when they are at different sites in the same animal. In certain cases, individual clones of cells from the same tumor have been found to express one or more distinct non-cross-reacting TATA.[23]

A given tumor cell may express more than one TATA. However, recent results[24] suggest that at least some of the TATA cross-react among different tumors and the number of different TATA is probably not large. In the context of the present review, an interesting feature of the TATA of chemically induced mouse (but not of rat) tumors is their inability to induce a humoral response in the host. However, such TATA of mice (and other species) may elicit an antibody response in a foreign species and thus provide polyclonal antibodies and MAB for targeting. The TATA of chemically induced rodent tumors differ from oncogenic virus-induced TATA in that tumors induced by the same or closely related viruses share a common TATA.

Distinctive antigenic moieties like the TATA of chemically induced rodent tumors have not been found on human tumor cells. In the context of antigenicity, Weiss[20] has described two categories of "spontaneous" human tumors (i.e., tumors that arise without experimental manipulation irrespective of their etiological factors), namely those that arise in (1) severely immunosuppressed individuals and (2) immunocompetent individuals. Tumors induced by oncogenic viruses predominate in the first group. A variety of inciting agents, e.g., cigarette smoke, ultraviolet (UV) irradiation, asbestos, and probably some viruses as well, may be implicated in the second group of tumors. Virus-induced tumor formation and its progression may involve the activation of one or a series of oncogenes leading to either amplified synthesis of a normal product or the expression of an altered product which may be immunogenic.[25-27]

In addition to the very small number of human tumors of possible viral etiology (e.g., Burkitt's lymphoma, nasopharyngeal carcinoma, and T-cell leukemia) in which various virus-associated antigens have been detected in the transformed cells,[28] there is also evidence that many human tumors synthesize substances that are either absent from or are produced in extremely limited amounts by the homologous normal tissue. These substances are termed tumor markers or TAA when they either provoke or serve as targets of immunologic reactivity. (In the present context, antibody response is more important than cell-mediated immunity.) Many human TAA are associated with retrodifferentiation and are the products of reexpressed fetal genes, i.e., the so-called oncofetal antigens. Some, such as CEA and AFP, may be present in normal serum and tissues in minute amounts. However, a variety of conditions, e.g., hyperplasia, may substantially increase the production of these or cross-reacting antigens. Furthermore, with the availability of panels of MAB directed against different epitopes of a given TAA, cross-reactivity of many TAA with normal cellular components has been observed (see later). There may also be aberrant expression of various differentiation genes in cells during neoplastic transformation. For example, many human tumors may secrete one or another polypeptide hormone even though the homologous normal tissues might not. Neoplasms that secrete a hormone, aberrantly or otherwise, can be excellent targets of agents linked to antibodies against that hormone, provided that the

possible loss of the hormone-producing normal cells can be compensated for by appropriate replacement therapy.

In the same way, antibodies against other normal tissue antigens (indigenous or aberrant) that are expressed in a tumor cell population can be used for targeting cytotoxic agents if the normal cell population expressing the antigen is not vital for survival. For example, prostate-specific acid phosphatase, if secreted by a prostatic carcinoma, can be a useful target provided that the antibody against it does not cross-react with the acid phosphatase in such vital tissues as renal tubular epithelium. Casein and lactalbumin, normally synthesized by mammary epithelium during pregnancy, can be aberrantly expressed in mammary carcinomas and can thus be useful targets. B-cell lymphoma/leukemias are usually monoclonal and therefore express Ig of a particular idiotype. Antibodies directed against the idiotype of the Ig associated with such monoclonal B-cell neoplasms are uniquely specific and have been used for drug-targeting and/or serotherapy of experimental and human tumors.[29] Altered activity of one or more glycosyl transferases observed in transformed cells can lead to the incorporation of aberrant sugar residues into cell surface-associated glycolipids or glycoproteins and thus change the antigenic profile of tumor cell surface components. Finally, the alloantigen(s) in choriocarcinomas (which are of fetal origin) may be regarded as a TAA.

Since the introduction of hybridoma technology, ever-increasing numbers of reports are appearing on the production of "specific" MAB against human tumors. The tumor specificity of most remains to be conclusively established. In the usual practice for characterization, the product of a given MAB-producing clone is screened against target tumor cells and a panel of normal tissues. Such screening procedures usually fail to detect antigen(s) in minor normal cell populations (especially when not easily accessible) and normal antigens that are expressed only at a certain stage of differentiation or a certain phase of the cell cycle. For example, MAB-defined Ca-l antigen, once thought to be specific for human epithelial malignancies, has now been found in a number of normal tissues (see later). In the following section, those human TAA that have potential for use in antibody-mediated drug-targeting will be discussed.

A. Pan-Cancer Antigen(s)

An antigen that is present in all or most malignant tumors, irrespective of their histogenesis and status of differentiation, will be of great help in the diagnosis and treatment of cancer. Extensive efforts have been made to obtain and characterize such pan-cancer antigens; however, few have withstood careful scrutiny of the claimed specificity. For example, the polypeptide antigen(s) claimed to be present in different types of carcinomas[30] has been found in many normal tissues, especially in non-keratinizing epithelium and mesothelium and in normal serum.[31-33] The MAB-Ca-1-defined antigen designated by Ashall et al.[34] as Ca was claimed to be expressed by the majority of malignant tumors and only in the normal epithelial lining of fallopian tubes and bladder. However, subsequent studies have revealed reactivity of the Ca-1 MAB with a variety of normal tissues (for example, see Woodhouse et al.[35]). Ca 19-9, another MAB-defined antigen claimed to be a marker of several different types of cancer (e.g., colorectal, gastric, and pancreatic carcinomas), has been demonstrated to be chemically identical with the sialylated Lewis a⁻ determinant of a monoganglioside and a mucin. It has been found in large amounts in the seminal plasma of healthy donors.[36] Very small amounts of a malignancy-associated sialylated glycoprotein were isolated by Bolmer and Davidson[37] from the perchloric acid-soluble fraction of serum from patients with various types of cancer. This "cancer glycoprotein" had a high affinity for wheat germ agglutinin and *Ricinus communis* agglutinin. It was claimed to be shed from tumor cell surfaces, to be not one of the major acute phase reactant glycoproteins associated with malignancy, and distinguishable from CEA and AFP by its molecular

weight and chromatographic behavior. However, whether this glycoprotein is indeed present on the cancer cell surface and its tumor specificity remain to be ascertained. Oncomodulin is a parvalbumin-like calcium-binding protein found in 85% of rodent and human tumors tested but is not detectable in normal adult tissues and therefore has potential as a tumor marker.[38] Methods for immunocytochemical detection[38] and RIA[39] of oncomodulin have been developed. These may be of help in studying its expression in various human tumors and normal tissues. Malignin and other cancer recognins are low molecular weight polypeptides which are produced by a wide variety of tumors. It has been suggested that malignin may be a general transformation protein and may thus be a useful pan-cancer antigen. It is antigenic and induces the production of a specific antibody in the tumor host. An MAB against malignin has also been produced,[40] but its tumor specificity and diagnostic and therapeutic usefulness need further investigation.

Thus, the tumor specificity of most of the pan-cancer antigens is open to question. However, it should be pointed out that most of the anti-tumor MAB usually react with more than one and sometimes a large number of different histological types of tumors. For example, MAB produced against a 48-kdalton peptide antigen of ovarian cancer reacted with 90% of ovarian cancer and 60% of other human tumors, both benign and malignant, but not with any normal adult tissue.[41]

B. Glycoconjugates in Cancer Cells

Many of the behavioral characteristics of cancer cells, i.e., decreased adhesiveness, loss of contact inhibition, invasiveness, increased growth, and immortalization, may be attributed to changes in cell surface components, especially carbohydrates.[42-46] Differences in monosaccharide content and linkage, sequence, branching, and configuration in cell surface-bound carbohydrates have been observed in a wide variety of experimental and human tumors compared to their normal tissue of origin and may offer one basis for the multitude of TAA specificities.[45-47] Details of the changes during malignant transformation can be found elsewhere.[45,48] Here, the emergence of unique carbohydrate moieties in tumors that might be exploited for drug-targeting will be considered only briefly.

Hakomori[45] has tentatively listed ten types of chemical changes in the carbohydrate moieties of glycolipids and surface glycoproteins on tumor cells compared to those on homologous normal cells. These changes are brought about either by blocked synthesis of normally existing carbohydrate chains or by neosynthesis as a result of activation of glycosyl transferases that are virtually absent from normal cells. The most commonly observed change in surface-bound glycoproteins in cancer cells has been the appearance of relatively high molecular weight glycoproteins replacing smaller ones, i.e., the Warren-Blick-Buck phenomenon.[49] Its structural basis has been attributed to "increased branching at the mannose units of complex oligosaccharides and/or increased sialylation of carbohydrate moieties".[45,48] Several MAB against human TAA are apparently directed against sialosyl residues of a densely glycosylated region of sialosyl-oligosaccharide-containing glycoproteins.[34,50-53] The differences from glycopeptides of normal homologous cells usually (but not always) disappear after treating high molecular weight glycoconjugates from cancer cells with sialidase.[48] From these and similar studies, it appears that the branching enzyme, *N*-acetyl glucosamine transferase, may play a crucial role.

There is no firm evidence that even tumors of a given histogenesis contain a common tumor-associated glycoconjugate or that the tumor cell surface contains unique carbohydrate sequences that are absent from all normal cells. Differences so far found are mainly quantitative, involving several membrane constituents. However, an increasing number of reports deal with the production of MAB that react with tumor-associ-

ated glycoconjugates that are present in all or a proportion of one or more given histological types of tumor and are fairly (though not absolutely) tumor specific. Hakomori[45] and Feizi[46] have also listed TAA, whose carbohydrate moieties have been defined by either classical chemical-immunological analysis or by use of MAB. The analysis of various human tumors has revealed the cessation of synthesis of long chain glycolipids leading to accumulation of immunogenic precursors that could be defined by MAB, e.g., G_{D3} in human melanoma[54,55] and acute nonlymphocytic leukemia,[56] G_{D2} in neuroectodermal tumors,[57] Gg3 in Hodgkin's lymphoma,[58] and Gb_3 in Burkitt's lymphoma.[59] Furthermore, MAB with highly restricted specificity have been obtained against a variety of fucolipids and fucogangliosides that have been observed to accumulate in various human cancers, especially those derived from endodermal epithelium, e.g., the gastrointestinal tract,[60-64] lung,[45] and mammary gland.[65] These antigens may result from activation of aberrant fucosyl or sialosyl transferases and may thus be regarded as the products of neosynthesis.

Another mechanism underlying the emergence of carbohydrate-containing neoantigens may be incomplete or blocked synthesis of glycolipids and glycoproteins. Precursor glycoconjugates, especially glycolipids, may accumulate in cancer cells as a result of blocked synthesis of more complex glycolipids. Reduction in size has been observed, especially in glycolipids,[48] although in transformed cells, the predominant change in cell surface-bound carbohydrates is increased size. New carbohydrate moieties appear in some tumors, whereas carbohydrate moieties cease to be synthesized in others. Decreased sialylation of tumor glycoproteins is also a fairly common observation.[66]

Alterations in the steric arrangement of the carbohydrate and other immunogenic moieties of cell surface-associated glycoconjugates may also play a role in the expression of TAA. Factors that are important in this context include: (1) concentration of epitopes per unit area of the cell surface and the uniformity of their distribution, i.e., the state of cluster; (2) contiguity of epitopes to masking moieties, e.g., other glycolipids and proteins; and (3) orientation and steric stability of the epitopes which will depend upon ceramide composition, cholesterol content, degree of unsaturation of the phospholipid acyl chains, and the protein/lipid ratio in the cell membrane.[44,45,67] Such TAA may be exploited for drug-targeting.

C. Blood Group Antigens as Tumor Markers

The alterations in the expression of blood group glycoprotein and glycolipid antigens in malignancy have been extensively reviewed by Hakomori[68] and Hakomori and Kobata.[69] These changes include: (1) deletion of host blood group antigen, (2) accumulation of precursors, and (3) emergence of new blood group determinants foreign to the host. Only changes under (2) and (3) above are pertinent to this review.

1. Precursor Accumulation

The core glycolipid structure that is the precursor to A, B, O blood group substances has been found at elevated levels in gastric and colonic adenocarcinomas.[70] The M, N blood group precursor antigens T and Tn have been found at elevated levels in about 90% of carcinoma tissues including breast, colon, and gastric carcinoma.[71] Most healthy human tissues, including fetuses, contain T and Tn antigens that are masked by sialic acid covalently linked carbohydrates and/or by tertiary structures and therefore are not immunoreactive. In contrast, both primary and metastatic adenocarcinomas and some squamous cell carcinomas contain immunoreactive forms of T and Tn antigens. The "unmasking" of these two antigens in tumor tissue may be brought about by the blocked synthesis of N or M antigens, increased activity of T and Tn synthesizing transferases, or excessive enzymatic desialylation in cancer cells. The desialylated antigens are treated as foreign by the tumor host and lead to elicitation of a

cell-mediated immune response.[71] Naturally occurring anti-T and anti-Tn antibodies are found in the serum of all humans, probably as a result of antigen stimulation from intestinal flora. This is in contrast to the lack of immunogenicity of oncofetal antigens. Circulating anti-T antibody binds to the unmasked T antigen on the surface of carcinoma cells and sheds cell surface components that contain T antigen. Human anti-T antibody in the presence of complement kills carcinoma cells in vitro. Apart from antibodies, T and Tn antigens bind to peanut lectin. Radioiodinated peanut lectin[72] and non-IgM MAB against T and Tn antigens[71] have been successfully used for the detection of T- or Tn-containing carcinoma xenografts in mice, a species that has no or little preexisting anti-T and anti-Tn antibodies.[71]

2. New Blood Group Determinants

Early studies by Hakomori[68] and other groups have revealed that: (1) tumor tissues may delete A and B determinants due to blocked activity of A and B transferases; (2) the type 2 chain may be fucosylated by enhanced activity of alpha 1 > 3 fucosyltransferase leading to the accumulation of Le^x hapten (or X-hapten) glycolipid in some tumors; and (3) the type 1 chain may be fucosylated by enhanced alpha 1 > 4 fucosyltransferase as well as alpha 1 > 2 fucosyltransferase to accumulate both Lea and Leb haptens, regardless of the blood group Lewis status of the host. More recent studies designed to elucidate the nature of human TAA that reacts with various antihuman tumor MAB (e.g., MAB against colonic, gastric, and pulmonary carcinomas and myelogenous leukemic cells) have revealed the antigenic determinant of these antitumor antibodies to be Le^x (or X) hapten.[73] However, Le^x is widely expressed in a variety of normal tissues (e.g., gastrointestinal and renal tubular epithelium, granulocytes, etc.) and therefore antitumor antibodies with Le^x specificity will have little usefulness for drug-targeting. Several more of the "antihuman tumor" MAB have been found to detect blood group determinants, e.g., an antipancreatic tumor antibody showing blood group B glycolipid specificity, an antihuman colon carcinoma antibody showing anti-A activity, antibodies against epidermoid carcinoma and antihuman lymphoid cell lines showing anti-H substance activity, and several MAB against human colonic, gastric, and pancreatic adenocarcinomas showing activity against antigens of the human Lewis blood group system.[68,74] Tumor-associated Lewis antigens may be useful markers for drug-targeting in cancer patients who are nonsecretors of Lewis substance since Lewis antigens are expressed in epithelial tumors, irrespective of the original secretor (Se) phenotype. Systematic analyses of human cancer cells with MAB have revealed novel dimeric and trimeric structures of X and its sialylated forms in human colonic cancer cells. MAB against these new antigens did not react with monofucosyl X or short-chain sialosyl X[68] and thus may prove to be fairly cancer specific.

Accumulated type 2 chain precursors have been detected as glycoproteins with Ii activity in two cases of gastric cancer, whereas accumulation of unsubstituted type 1 chain led to appearance of antigen moieties unrelated to Ii and the production of antitumor autoantibody in a patient with bronchogenic carcinoma.[68] There is also evidence that unbranched type 2 chain can accumulate in human leukemic cells, probably due to the blockade of synthesis of blood group determinants.[75]

Aberrant (or incompatible) blood group substances have been noted in various adenocarcinomas, e.g., the appearance of A-like antigen in 10 to 15% of gastrointestinal carcinomas of O and B group individuals.[76,77] This provides a possible model for the use of a patient's own anti-A antibody for drug-targeting. The chemical basis of the emergence of A-like antigen in their tumor cells is not very clear. It is possible that the aberrantly expressed A antigen in at least some patients is Forssman antigen since the latter cross-reacts with blood group A. Forssman antigens have been detected in human lung, gastric, and breast cancer cell lines.[68] Other rare examples of the aberrant ap-

pearance of blood group antigens in tumors include the detection of P-like or P_1-like antigens in tumors of a patient with pp genotype[68] and the appearance of a MAB-defined Burkitt's lymphoma-associated antigen, i.e., a globotriaosylceramide.[78] It appears that Burkitt's lymphoma lymphocytes express the pK antigen irrespective of the P blood group status of the patient. This antigen is present in a large variety of normal cells but is not expressed on the surface.

D. Tumor Markers Used for Targeting

Some of the common human tumor markers and associated tumors are described below. Tumor markers have been classified into three somewhat overlapping groups, i.e., (1) products of activation of fetal genes, (2) products of ectopic activation in tumors of genes involved in the synthesis of pregnancy-associated proteins, and (3) adult or fetal tissue-specific enzymes or isozymes that may be synthesized in tumor cells. In addition to their use for tumor identification and detection, antibodies to many have been used for drug or radionuclide targeting and the following section outlines markers that have been used or have potential for targeting.

1. Products of Expression of Fetal Genes

a. Carcinoembryonic Antigen (CEA)

CEA is synthesized by a large variety of epithelial tumors, especially cancers of the colon and rectum, breast, pancreas, stomach and lung, and by a proportion of cancers of the prostate, bladder, ovary, uterus, and thyroid. It has also been found in neuroblastomas and osteosarcomas. The term CEA designates a heterogeneous group of rather complex glycoproteins; the protein-to-carbohydrate ratio in CEA from different tumors has been found to vary from 1:5 to 1:1. Polyclonal antisera against CEA cross-react with a number of normal tissue antigens, including several biliary glycoproteins, an antigen in normal feces, colon, and normal gastric juice.[79,80] Cells in a number of benign lesions produce CEA, e.g., hyperplastic gastrointestinal epithelium in ulcerative colitis and Crohn's disease, intestinal polyps, benign prostatic hypertrophy, bronchitis, etc.[81,82] With the availability of MAB, a paramount question is whether there are epitopes in the CEA molecule that are specific for cancer. Accolla et al.[83] reported the production of two anti-CEA MAB that had very limited cross-reactivity with normal colon antigens. Most of the 20 anti-CEA MAB studied by Grunert et al.[84] could not distinguish epitopes expressed in CEA from epitopes expressed in other cross-reacting molecules. On the other hand, by studying the pattern of reactivity of eight anti-CEA MAB, Hedin et al.[85] were able to conclude that there are at least six different epitopes in the peptide moiety of CEA. Two of the MAB cross-reacted with normal colon antigens but none with biliary glycoproteins. The results of Primus et al.[86] on the reactivity of their anti-CEA MAB are consistent with the pattern of epitopes suggested by Hedin et al.[85] In the context of their carrier role, three points should be made about anti-CEA MAB: (1) the pattern of reactivity of anti-CEA MAB obtained after immunization with tissue preparations other than colorectal cancers suggests that CEA preparations from histologically different tumors contain epitopes specific for that type of tumor;[87] (2) at least some of the anti-CEA MAB show no additive binding when the various antibodies are mixed, indicating that one or more can react with a spectrum of antigenic sites;[88] and (3) some of the anti-CEA MAB have very low affinity and low binding capacity for CEA compared to polyclonal antibodies of goat or rabbit origin, even when the former shows a degree of specificity for CEA.[88,89] In a study using three MAB directed against three different epitopes of CEA and xenografts of three different CEA-producing human tumors, factors that determined tumor-specific localization were found to include the concentration of CEA in the tumor and the target epitope of the MAB.[85,90] An ^{131}I-labeled polyclonal anti-CEA antibody inhibited the growth of

a CEA-producing human tumor xenografted in hamsters.[91] MAB directed against certain epitopes of CEA have been found to cross-react with antigenic moieties on circulating cells. These MAB did not localize in the target tumor and caused systemic toxicity.[92]

In the context of anti-CEA antibody-mediated targeting of drugs, expression of CEA by hyperplastic or benign epithelial lesions does not constitute a problem. On the other hand, high levels of circulating CEA and cross-reactivity of anti-CEA antibodies with normal cells limit the use of this tumor marker for drug-targeting. Production by tumors usually elevates the level of the antigen in circulation. Although it has been claimed that circulating CEA does not prevent the binding of anti-CEA antibody to CEA-producing tumors in vivo,[93] the possibility remains that elevated levels of circulating antigen will interfere with the binding of anti-CEA antibody to the target tumor and may give rise to circulating immune complexes. Radiolabeled anti-CEA antibodies have been used for the imaging of various human carcinomas[93] and as carriers of cytotoxic agents (see Ghose et al.[14]).

b. Alpha Fetoprotein (AFP)

AFP is produced by hepatocellular cancers, gonadal and extragonadal germ cell tumors (especially those that contain yolk sac elements), and cancers of the stomach, pancreas, and lungs. AFP from various types of cancer is much less heterogeneous than CEA and is antigenically identical to fetal AFP. It is synthesized by proliferating liver cells, especially during hepatocellular regeneration. AFP production by tumors usually leads to the elevation of serum levels. Anti-AFP antibody has been found to inhibit the growth of AFP-producing tumors in vitro, of rodent tumors in vivo, and of AFP-producing human tumors in nude mice.[82,94] Anti-AFP antibodies have been used for radioimmunodetection of cancer[93,94] and as carriers of cytotoxic agents.[14] A number of anti-AFP MAB have been produced (listed recently by Sell[94]). Four epitopes have been identified on AFP with the use of panels of these MAB.[95] Most anti-AFP MAB appear to have less affinity than their conventional polyclonal counterparts.

2. Pregnancy Proteins

These proteins (or antigenically closely related substances) may be produced by trophoplastic tumors or ectopically by various nontrophoplastic tumors.

a. Human Chorionic Gonadotropin (hCG)

Production of hCG occurs in trophoblast and germ cell-derived tumors and also ectopically in a proportion of other tumors, e.g., ovarian, breast, gastrointestinal, liver and lung cancers, melanomas, islet cell tumors, etc. In addition to trophoblastic tissue, small amounts of hCG are present in testis, pituitary, and gastrointestinal tissue.[96,97] The alpha chain of hCG is shared by LH and other glycopeptide hormones. Although a portion of the beta chain (i.e., N-terminal amino acids 116 to 145) is responsible for its antigenic identity, the beta subunit also cross-reacts with other glycopeptide hormones, especially LH. Antisera raised against the unique carboxy terminal peptide of the beta subunit have essentially no cross-reactivity with hLH.[98] A large number of MAB have been produced against hCG and its subunits.[99] These show a wide range of affinity and cross-reactivity. There appear to be four major epitopes each in the alpha and beta subunits. In regard to the carrier role of anti-hCG MAB, it is interesting that certain pairs of MAB display synergism in binding. The mechanism involves the formation of a circular complex consisting of two antigen and two antibody molecules.[99] Synthesis of hCG by tumors also is usually accompanied by an increase in the level of hCG in circulation. Furthermore, there is usually an increase in the circulating level of LH in gonadectomized patients. Elevated serum levels of hCG or LH, that cross-react

with anti-hCG antibody, might interfere with the tumor localization of the antibody. Some tumors produce only isolated free subunits of hCG that might not react with a given antibody.[97,100] Antibodies to beta-hCG have been used for tumor imaging.[93]

b. Placental Lactogen (hPL)

Apart from trophoplastic tissue, hPL is produced in small amounts by normal testis. It is produced by both male and female choriocarcinomas and a large proportion of breast cancers.[101] Compared to hCG, the serum level of hPL is usually lower in most patients. With residual tumor, hPL becomes undetectable in serum, whereas hCG can still be demonstrated.[102] Thus, for drug-targeting, hPL may be a more suitable target, provided that adequate amounts are produced by target tumor cells.

c. Pregnancy-Specific Glycoproteins

These include SPI alpha and SPI beta (synonyms: trophoblast-specific beta globulin [T BGi]; pregnancy associated plasma protein C [PAPP-C]; and pregnancy-specific beta, glycoprotein). In addition to trophoblastic tumors, immunoreactive SPI has been found in 60% of breast cancers and 50% of malignant gastrointestinal epithelial tumors.[101] They have also been found in the breast duct epithelium in a proportion of patients with benign breast disease.[97] In both trophoblastic and nontrophoblastic tumors, the serum levels of SPI have been found to be low compared to that of hCG. After chemotherapy, circulating SPI disappears much earlier than hCG.[103] Thus, there may be less interference with the carrier antibody by circulating SPI.

d. Placental Protein 5 (PP5)

PP5 is present in small amounts in the cytoplasm of syncitial trophoblasts and in the cytoplasm of breast carcinoma cells.[97] Adequate studies have not been carried out to establish its presence in other tumors or in the serum of cancer patients.

3. Tissue-Specific Enzymes

a. Acid Phosphatase (E.C.3.1.3.2)

Prostatic carcinomas contain different proportions of normal isozymes of acid phosphatase.[104] The serum level of PAP is raised both in prostatic carcinoma and benign prostatic hyperplasia. Antibodies to PAP cross-react with acid phosphatase in other tissues (i.e., liver, spleen, bone, kidney platelets, leukocytes, and erythrocytes).[104] Antibodies to PAP have been successfully used for imaging human prostatic carcinoma.[17,93]

b. Alpha Lactalbumin

Alpha lactalbumin is exclusively present in mammary tissue and its level goes up in mature breast tissue as a result of prolactin stimulation. It is the B protein of lactose synthase (E.C.2.4.1.22). The A protein is present in a higher proportion of breast cancers than the B protein but occurs in various other tissues. The B protein has also been found in the serum of breast cancer patients.[105]

c. Alkaline Phosphatase (E.C.3.1.3.1)

All alkaline phosphatase variants associated with cancer have counterparts related to early fetal development, i.e., these are fetal isozymes recurring in cancers. Various human cancers such as hepatomas produce the Regan or Kasahara isozyme.[105] It closely resembles the B variant found in human placenta. This isoenzyme is ectopically expressed on the surface of tumor cells in 12% of different types of carcinoma but in over 50% of ovarian carcinoma and seminoma.[106] There is elevation in the serum level in patients who have these isozyme-producing tumors.[105] A MAB against this isozyme

has been successfully used to target ricin A chain against a human cervical carcinoma line in vitro.[107]

d. Alcohol Dehydrogenase Isozymes (E.C.1.1.1.1)

In some hepatomas there is reexpression of the early fetal form of isozyme aa.

e. Creatin Kinase (CPK) (E.C.2.7.3.2)

The isozymes of CPK are fairly tissue specific. MM and BB are the only isozymes in muscle and brain, respectively. BB predominates in fetal tissues and adult gastrointestinal tract, uterus, and prostate. Tumors continue to produce the isozyme typical of the tissue of origin, although there may be reexpression of the embryonic form BB.[105] In patients, there is usually elevation of the serum level of the isozyme produced by the tumor.

f. Salivary Amylase (E.C.3.2.1.1)

Some lung and ovarian cancers produce salivary amylase, whereas only small amounts are produced by normal lung tissue. Amylase produced by tumors may differ slightly from salivary amylase in its carbohydrate moiety.[105]

g. Pancreatic Amylase

This amylase is produced by cancer of the pancreas, leading to elevation of serum amylase in patients.

E. Lymphoma/Leukemia Markers

The use of various polyclonal and MAB to study the cell surface antigen(s) in lymphomas/leukemias has provided insight into the ontogeny, differentiation, and function of various subpopulations of cells in the lymphoid/hematopoietic systems.[108] Precise schemes of T-cell differentiation and function have been delineated with MAB reagents,[109,110] but markers of B-lymphocyte differentiation have not been as well defined. Antibodies to HLA-DR[111] and to surface Ig were the principal reagents, but recently a number of MAB to other B-cell differentiation antigens have become available. Several excellent reviews have appeared on the surface markers of both normal and malignant T, B and other lymphoid and hemopoietic cell populations, and the various MAB that help in their identification.[112-117] Characterization of the differentiation antigens on T- and B-lymphocytes shows that many of the leukemia-specific or leukemia-associated antigens identified with polyclonal antibodies are in reality differentiation antigens.[116,117] For example, CALLA, originally defined with rabbit antisera after immunization with SIg^+, ER-ALL cells, and more recently with MAB J5, is present in about 70% of patients with all types of ALL and also in lymphoid B-cell precursors in normal bone marrow.[117] Several of these "antileukemia" MAB have been used in the serotherapy of leukemia in spite of their lack of specificity.[118] Most MAB against nonlymphoblastic leukemias only detect lineage-restricted normal hematopoietic differentiation antigens, and there is no evidence that these MAB can distinguish leukemic stem cells from normal pluripotent hematopoietic stem cells.[119]

Several MAB appear to demonstrate a fair degree of tumor specificity. For example, Naito et al.[120] have described two (MAB 3-3 and 3-40) that reacted with T-ALL cells but not with normal hematopoietic cells, and Negoro and Seon[121] obtained several that reacted with both acute and chronic T-leukemia cells. Berger et al.[122] reported MAB BE-1 and BE-2 that reacted with two different antigens on cutaneous T-cell lymphoma but not on normal T-cells. Virus-associated antigens of HTLV1 appear to be a potential tumor-specific target for MAB. Several MAB are available for HTLV internal core proteins including p19 and p24 HTLV proteins that appear to be membrane associated,

i.e., either on the inner surface of the T-cell membrane or inside HTLV on the infected T-cell surface (see Harden and Haynes).[117] Also, MAB that are directed against proteins outside the virus core are becoming available, e.g., MAB HT462 that is specific for an antigen expressed by HTLV and by HTLV-infected cells.[123] As already stated, the idiotype of the Ig molecule on B-cells offers a very specific target.[29]

F. TAA in Solid Tumors

In this section, without being comprehensive, information on human TAA that have been fairly well characterized (and therefore can be used for the production of well-defined anti-TAA antibodies) and some of the MAB against human TAA that appear to be potentially useful for drug-targeting will be briefly summarized. Two more comprehensive reviews on MAB against human TAA have been published recently.[124,125] Evidence for the specificity of most MAB-defined human TAA is inadequate. Many are oncofetal or differentiation antigen(s), while others merely reflect quantitative changes rather than *de novo* synthesis of antigenic moieties. Use of sensitive assay systems and careful examination of various normal cell populations at different stages of differentiation and different phases of the cell cycle have raised the question of whether human tumor antigens with unique specificity really exist.[126]

It has been proposed[127] that MAB-defined TAA can be classified into three categories: class 1 antigens that are restricted to autologous tumor cells (i.e., individual tumor specific); class 2 antigens that are common to histologically similar tumors from several patients but absent from other tumors and normal tissues; and class 3 antigens that are common to tumor cells and various components of normal tissue. Antibodies against class 2 antigens appear to be the most useful for targeting. In regard to class 1 antigens, every tumor has to be carefully analyzed serologically for the detection of the unique TAA. This is likely to be a time-consuming process jeopardizing therapeutic effectiveness. Most anti-TAA antibodies (irrespective of whether they are polyclonal or monoclonal in origin) appear, on close scrutiny, to be directed against class 3 antigens. However, this is not a serious limitation of their usefulness as carriers. A substantial quantitative difference in the distribution of the target antigen between the tumor and surrounding normal tissue may provide an adequate gradient for the localization of the carrier antibody, provided that the presence of cross-reacting antigen in the circulation or any other accessible tissue does not divert the antibody away from the tumor. Furthermore, localization of cytotoxic-agent-antibody conjugates in TAA-bearing non-vital normal tissue(s) may not be an insurmountable limiting factor.

1. Malignant Melanoma

Interest in the immunotherapy of human melanoma has led to extensive investigations on human MAA using both polyclonal antibodies[128,129] and MAB. More than 30 different MAB against MAA have been produced.[129-135] Most of the MAB-defined MAA are proteins but some are glycoproteins, proteoglycans,[129] or glycolipids.[134] The p97 MAA appears to be closely related to transferrin.[130,136] The list of MAA includes DR and other alloantigens, nerve growth factor receptors, and antigens shared by melanomas, nevus cells, tumors of neural crest origin, and various other tumor and normal fetal or adult cells.[134,137] However, with the probable exception of MAB D 1.1 (directed against aberrantly O-acetylated GD_3 diasialoganglioside),[138] no MAA with exclusive melanoma specificity have been identified using MAB.[134] Nevertheless, a number of anti-MAA have been demonstrated to selectively localize in human melanoma in nude mice and patients.[16,139-143] There was also inhibition of growth of human M21 melanoma cells in nude mice after treatment with MTX conjugated to MAB 225.28S.[144] MAB of human origin have also been produced against ganglioside GD2,

a membrane ganglioside present on human melanomas, retinoblastomas, and neuroblastomas.[145]

2. Cancers of the Gastrointestinal Tract

Radiolabeled anti-CEA antibodies have been widely used for tumor detection in vivo[146,147] and as carriers of cytotoxic agents.[14] Other potentially useful markers of carcinomas of the gastrointestinal tract include the following.

a. Fetal Sulfoglycoprotein Antigen (FSA)

FSA is found in fetal gastrointestinal tract mucosa and gastric cancer. It is not usually found in normal adult gastric tissue but may be present in the involved gastric mucosa in diseases such as atrophic gastritis, peptic ulcer, gastritis, and polyps. Investigations suggest that FSA is a component of CEA of colonic origin.[148]

b. Gastric Carcinoma Sulfoglycoprotein Antigen (SGA)

This antigen is present in fetal intestine, adult normal colonic mucosa, and most commonly in well-differentiated mucinous adenocarcinomas but not in undifferentiated gastric adenocarcinomas.[149]

c. Glycolipids of Gastric Cancer

In general, human gastric cancer tissue contains more neutral glycolipids than does normal mucosa. Glycolipids of the lactose series, including fucolipids, are markedly increased in cancer. Blood group-A-like substances have been found in the tumor tissue of two O group patients but not in surrounding uninvolved mucosa.[150]

d. Fetal Gut Antigen (FGA)

Smith and O'Neill[151] obtained an antibody against a FGA which reacted with 87% of fetal gut extracts, 30% of gastrointestinal carcinomas, and 8% of normal gut tissues but not with the serum of patients with gastrointestinal cancer. Therefore anti-FGA antibody, in appropriate patients, could be a better carrier of cytotoxic agents than anti-CEA antibody. Unfortunately, FGA and several other similar antigens were mainly defined by polyclonal antibodies and have not been adequately characterized biochemically. Furthermore, how different these oncofetal antigens are from CEA is open to question.

e. Zinc Glycinate Marker (ZGM)

Pusztaszeri et al.[152] identified a 2-kdalton antigen with an alpha-2 electrophoretic mobility in well-differentiated adenocarcinomas of the colon. This antigen has been found also in gastric, breast, prostatic, and lung cancers. However, the antigen was found in normal colonic tissue in patients with colonic cancer and nonmalignant gastric, pyloric, and small bowel mucosa.

f. Colon-Specific Antigen P (CSAp) and Colon Mucoprotein Antigen (CMA)

Radiolabeled antibody to CSAp[153] could localize xenografts of a human colon carcinoma in hamsters better than anti-CEA antibody.[154] Goldenberg's group has also reported alterations in the antigenicity of a normal colon-specific mucoprotein antigen (CMA) in colonic adenocarcinomas.[148]

g. MAB-Defined Antigens

Steplewski and Koprowski[155] have listed the various human colorectal carcinoma antigens defined by MAB. These are either glycoproteins (a few being identical with CEA), neutral glycolipids, monosialogangliosides, or members of the Lewis blood

group antigens. None are strictly colorectal carcinoma specific and a few react with erythrocytes or granulocytes. MAB 19.9 is directed against a ganglioside with a sialylated Lewis A carbohydrate.[137] This TAA is expressed by adenocarcinomas of the gastrointestinal tract, including pancreas, and by intestinal polyps. It is present only in a single layer of epithelial cells lining bronchi, pancreatic ducts, etc. in the saliva of Le^{a+b-} and Le^{a-b+} individuals and in meconium. Although this TAA is present in the serum of patients with gastrointestinal cancer, radiolabeled MAB 19.9 has detected gastrointestinal cancers.[156]

3. Liver Tumors

AFP has been the most used marker of human hepatomas. Antibodies to AFP have been used for radioimmunodetection of AFP-producing tumors and as carriers of cytotoxic agents in experimental tumor models (see Ghose et al.[14]). Immunohistological methods have demonstrated hepatitis B virus antigen in the liver tissue of most hepatoma patients. However, it is most abundant in cells that do not appear to have undergone malignant transformation.[157]

4. Pancreatic Carcinoma

Due to the difficulties in early diagnosis, there has been an extensive search for clinically useful markers of pancreatic cancer. CEA has been fairly widely used for this purpose. However, the presence of CEA or antigenically similar substances in other cancers and in normal and certain pathological tissues has severely limited its usefulness as a specific marker of pancreatic cancer.[158] Using homogenates of whole fetal pancreas, Banwo et al.[159] claimed to have obtained a polyclonal serum that could detect a pancreas-specific oncofetal antigen, i.e., pancreatic oncofetal antigen (POA). Though initially POA was found to be elevated only in the serum of patients with cancer of the pancreas, more extensive studies revealed elevated serum levels in other cancers and even in normal individuals with liver disorders.[160] In many of the earlier investigations, POA was not biochemically characterized and it was not even certain whether the different batches of polyclonal sera used in these studies were detecting the same antigen. Recently, two pancreas-specific antigens, i.e., one designated PaA (pancreas-specific antigen, a single chain protein of molecular mass 44 kdaltons)[161] and another, a pancreatic duct mucin-specific antigen,[162] have been defined by polyclonal sera. Both are expressed by at least a proportion of human pancreatic adenocarcinomas. Several TAA of human pancreatic carcinomas have also been identified by polyclonal antibodies[160,163] and MAB.[53,164-167] Most pancreatic TAA lack specificity, e.g., the heavily glycosylated mucin-like antigen detected by DU-PAN-2 is present on normal pancreatic ductal epithelium.[164] MAB AR2-20 and ARI-28 (directed, respectively, against 190- and 10-kdalton moieties)[167] and MAB C-P83 (directed against a 100-kdalton moiety)[168] appear to be fairly tumor specific after somewhat limited screening. However, the serum level of both organ- and tumor-associated pancreatic antigens is elevated in most patients with cancer of the pancreas.[161,169] For a more detailed description of MAB- and polyclonal antibody-defined tissue and tumor-associated antigens of the pancreas, see Sell and Reisfeld.[124]

5. Breast Tumors

As with other carcinomas, CEA has been the most used marker of mammary carcinomas. In a search for more reliable markers, Imam[170] has evaluated the potential clinical usefulness of various breast cancer-associated markers. These include: (1) ectopic products of breast tissue, e.g., a lactalbumin, casein, lactoferrin, and the milk globule membrane; (2) oncofetal antigens, e.g., CEA, beta oncofetal antigen (OFA); (3) hormones, e.g., hCG, calcitonin, and steroid receptors;[171] (4) enzymes, e.g., Regan

isotype of alkaline phosphatase, sialyl transferase, etc.; (5) pregnancy-associated glycoproteins;[172] (6) ferritin; (7) T-antigen; (8) nucleosides; (9) polyamines; (10) viral proteins. None appear to have adequate specificity or sensitivity for diagnostic or therapeutic purposes. Earlier investigations using autologous sera from patients demonstrated that there were antibodies that reacted with antigen(s) in breast tissue in a large proportion of breast cancer patients. However, the nature of these antigens and their tumor specificity were not adequately established. Later attempts at isolation yielded TAA, most of which showed substantial cross-reactivity with CEA.[148]

Increasing numbers of reports are appearing on MAB that react with human mammary carcinomas. They have been classified into four groups on the basis of their method of production and the immunogen used. One procedure uses lymphocytes from lymph nodes draining breast cancer, i.e., B-cells sensitized against TAA in vivo. Several of these MAB could discriminate between normal and malignant mammary epithelium.[172] However, all showed reactivity with epithelial cells lining renal tubules and sebaceous glands and therefore their therapeutic potential appears to be limited. Other procedures use splenic cells from mice immunized with human breast cancer cell lines, milk fat globule membrane, or crude or membrane-enriched fractions of primary or metastatic breast cancer. Schlom et al.[173] have listed the available MAB against human breast cancer and the nature of the antigen with which they react. Although it has been claimed that some react specifically with mammary cancer cells[174,175] or with both normal and neoplastic mammary epithelium,[176] none are specific for breast cancer and all appear to be antibodies against class 3 TAA. Furthermore, every MAB behaves differently with respect to "percentage of reactive mammary tumors, percentage of reactive cells within tumors, cellular location of the TAA, and extent of reactivity with non-mammary tumors and normal tissues".[173] In spite of these limitations, specific localization of radioactivity in breast cancer has been demonstrated after administration of radiolabeled polyclonal antibodies (or their reactive fragments) against CEA[177] or hCG[178] and radiolabeled MAB against mammary cancer (see Schlom et al.[173]).

6. Prostate and Prostatic Carcinoma

A number of human prostate-specific antigens including PAP have been isolated and several have been biochemically characterized.[104,179] As carriers of cytotoxic agents in the treatment of primary and metastatic carcinoma of the prostate, antibodies to prostate-associated antigens are likely to be useful provided that these normal tissue markers are adequately expressed by the tumor tissue. The tissue-specific antigens, prostatic antigen (PA) and PAP are produced by the majority of primary and metastatic prostatic carcinomas, although PAP production may be reduced in tumor tissue, especially if the tumor is undifferentiated.[104,180,181] The serum levels of these markers are elevated in patients with prostatic cancer. Several MAB have been obtained against different prostatic TAA[180,182,183] and normal PA,[184] including PAP.[104,185] MAB against different epitopes of PAP[185] and a prostatic TAA[186] have also been obtained. MAB D83.21 against prostate carcinoma also reacts with human bladder cell lines,[182] and the antigen recognized is a membrane glycoprotein with 60- and 28-kdalton subunits cross-linked through disulfide bonds.[187] Both polyclonal antibodies and MAB against PAP have been demonstrated to localize in xenografts of human prostatic carcinoma.[17,188] It has been claimed that anti-PAP MAB-linked fluorouracil deoxyriboside inhibited the growth of a prostatic carcinoma xenograft in nude mice.[188] Although the serum levels of PAP, prostate-specific, and prostatic TAA are usually elevated in patients with prostatic cancer, the serum levels of all the markers are not raised in all patients.[185,189] For agents that need endocytosis, it is pertinent that the cell surface-located prostatic TAA moiety P54 is not shed but internalized after binding to MAB alpha

Pro3 or alpha pro5. These two MAB bind to P54 via different epitopes and show a synergistic effect on endocytosis.[186]

7. Carcinoma of the Ovary

Adenocarcinoma of the ovary is the most lethal gynecological cancer and its curability falls sharply with each advance in the stage of the disease. Both AFP and hCG have been widely used as markers of ovarian germ cell tumors. AFP is associated with endodermal sinus tumors and embryonal carcinomas, while hCG is associated with choriocarcinomas and embryonal tumors but not with endodermal sinus tumors. CEA is not a useful marker for most of the epithelial tumors.[190] Before the advent of hybridoma methodology, autologous sera from patients with adenocarcinoma of the ovary or xenogenic immune sera were used to define putative ovarian carcinoma-associated antigens. Except for one,[191] they have been poorly characterized and a few proved to be identical with CEA.[148,190] Bhattacharya et al.[192] identified six different ovarian carcinoma-associated antigens in ovarian cytoadenocarcinoma. Other ovarian TAA include the glycoproteins OCA[193] and CA 125.[194] However, most of the antibodies cross-react with fetal or adult intestine[192] and/or other tumors.[195,196] MAB DU-PAN 2 raised against pancreatic carcinomas reacts with approximately 40% of epithelial ovarian tumors. The CA 19-9 antigen, originally detected in a colorectal carcinoma cell line, has a sialylated Lewis blood group A determinant on a mucin-like glycoprotein (Mr > 500 kdaltons) and CA 19-9 is found in approximately 40% of ovarian carcinoma tissues and in the serum of 20% of ovarian carcinoma patients.[190] The serum level of other ovarian TAA is usually elevated in these patients as well as in a proportion of patients with other nonovarian adenocarcinomas.[197] Radiolabeled antibodies against CEA and ovarian TAA have been used for imaging and treating ovarian cancer.[198-202]

8. Lung Cancer

Early attempts at the identification of TAA of lung cancer using immune xenoantisera led to the recognition of several antigens that were distinct from CEA, AFP, and ferritin, e.g., antigens X and Y[203], HLTAA, LTA I, and LTA II.[204] However, most were not adequately characterized or properly evaluated in regard to their specificity.[148,205] It is now realized that human lung cancers may be divided into two broad categories, i.e., small cell lung cancers (SCLC) and non-SCLC that include squamous, adenocarcinomas, and large cell cancers. SCLC and bronchial carcinoids are related to or arise from pulmonary endocrine cells having APUD properties (i.e., cells that have the ability for amine and precursor uptake and decarboxylation) and therefore it is not surprising that many eutopic and ectotopic hormone markers are produced by SCLC tissue. These include the APUD cell marker, L-dopa decarboxylase, neuron-specific enolase, the specific peptide product bombesin, enkephalins, and calcitonin. Other peptide products elaborated most often by SCLC (and occasionally by non-SCLC) include arginine, vasopressin, and neurophysial oxytocin. Single SCLC tumors have been reported to secrete up to ten hormones. SCLC also produce non-APUD markers such as CPK and its BB isoenzyme.[205,206] In contrast to SCLC, which are most frequently associated with APUD cell products, non-SCLC are associated with both APUD and non-APUD hormones. For example, although clinical syndromes due to excessive production of ACTH secretion have been limited to SCLC and bronchial carcinoids, all types of lung cancer produce proACTH and lipotropin, a large protein that is a precursor to both proACTH and ACTH. Furthermore, all types of lung cancer cells secrete calcitonin and hCG. Some hormones are synthesized more commonly in non-SCLC than in SCLC, e.g., human placental lactogen and growth hormone. There is a frequent association between the bizarre giant cell type of large cell carcinoma and hCG secretion.[205]

Immunochemically, the hormones produced by malignant cells may be heterogeneous, e.g., calcitonin,[207] or may consist of various free subunits, e.g., hCG. In the context of tumor markers, SCLC tend to produce ectopic polypeptide hormones such as ACTH and bombesin and are rich in neuron-specific enolase, L-dopa decarboxylase, and creatine phosphokinase BB.[206] MAB have been produced that react with both SCLC and non-SCLC,[208] with non-SCLC only,[206] with mainly squamous cell carcinomas,[209] or with human cancer cell lines.[210,211] The MAB 534 F8 against SCLC generated by Cuttitta et al.[208] recognizes lacto-*N*-fucopentose III. This oligosaccharide is expressed in murine embryos and embryonic carcinomas (SSEA-1 antigen), normal human bronchial epithelium and myeloid cells, and various proportions of other human cancers, e.g., NSLC, colon and breast cancers, and malignant cells of Hodgkin's disease.[124] Other TAA found in lung cancers of diverse histology are CEA-related antigens and an antigen that inhibits alpha-1-chymotrypsin.[212]

Recently, several MAB with more restricted reactivity have been obtained, e.g., MAB that react with lung tumors of a given histologic type such as squamous cell carcinoma, adenocarcinoma, and large cell carcinoma but not with SCLC, other human tumors, and normal tissues.[213] MOC-1 reacts with SCLC and normal Kulchitski cells that constitute a subset of normal endocrine and neural cells.[214] MAB, TFS-2, and SM-1 that, along with complement, specifically lyses SCLC cells but does not react with normal lymphoid or bone marrow cells have been successfully used to purge SCLC cells from human bone marrow.[215,216] However, there is considerable heterogeneity of antigen expression in lung tumors of even a given histologic type.[124] An IgM MAB, 600 D11, selectively localized in human SCLC xenografts in nude mice.[217] There is some evidence that MAB 2i1 (that has specificity for bombesin) and MAB 11G11 (that reacts with a subset of SCLC) inhibits target tumors in vitro and their xenografts in nude mice.[124]

9. Osteosarcoma

An MAB that reacts with several different human osteogenic sarcoma lines has been successfully used to image human osteogenic sarcoma xenografts in nude mice. Conjugates of this MAB with MTX, vindesine, adriamycin, or ricin A chain inhibited target cell lines.[218] However, it cross-reacts with other human tumor lines that are histologically different and also with some normal human tissues. Biochemically, it appears to be directed against an integral membrane protein of 72 kdaltons.[219] Hosoi et al.[220] have also produced several MAB that react with both human osteosarcoma and chondrosarcoma. Prior to this, appropriately absorbed polyclonal xenoactisera were shown to react with human osteosarcoma cells with varying degrees of specificity.[221]

10. Renal Cancer

An antibody against human renal cell carcinoma (RCC) was obtained by Ghose et al. after immunization of a RCC patient with a histologically similar allogenic tumor.[222] Ravitz et al.[223] also detected autoantibodies to RCC in the serum of RCC patients. By immunizing goats and rabbits with viable RCC cells and absorbing the immune sera with AB Rh+ human red cells and a panel of normal human tissues including kidney, Ghose et al. obtained antibody preparations that reacted with a large proportion of human RCC from different patients but did not react with any normal human tissues or with other histological types of human cancers.[224] Ghose et al. have also demonstrated that, after i.v. injection, these polyclonal anti-RCC antibodies selectively localized in tumor tissue.[225] Attempts have been made to produce RCC-specific MAB.[226-229] But, with rare exceptions,[226a] these antibodies cross-react with various other human tumors and normal tissues. The antigen detected by the MAB of Ueda et

al.[226] has been found to be the adenosine deaminase-binding protein.[230] Other TAA in RCC that can possibly be exploited for drug-targeting include renin[231] and steroid receptors.[232]

11. Urinary Bladder Cancer

As with renal cancer, investigations using either xenogenic antiserum[233] or autologous sera from bladder cancer patients[234] have suggested the existence of TAA in human bladder cancer. Fradet et al.[235] have used MAB derived from mice immunized with human bladder cancer cell lines or lysates of bladder papilloma to define 11 distinct antigenic systems. Two of these antigens, Om5 and J233, are not expressed by any cultured cells of normal origin or by normal and fetal tissues. Two other antigens, T101 and JP165, are subset markers for bladder cancer and are not detected in normal tissues. The remaining antigens are expressed by various other normal and neoplastic cells. All antigens detected by these MAB are heat labile and are not related to A, B, H, I, or Lewis blood group antigens.

12. Cervical Cancer

A number of TAA associated with squamous cell carcinoma of the cervix have been described using polyclonal xenoantibodies. Levi[236] obtained two precipitin lines after immunodiffusion of cervical carcinoma homogenates against a normal human tissue-absorbed rabbit immune serum obtained after immunization with homogenates of human cervical carcinoma. Levi's work stimulated a number of similar investigations with comparable results.[148,237] However, the biochemical nature of these antigens, the extent to which they are tumor or tissue specific, and the relationship among them remain controversial. Other TAA associated with cervical cancer include CEA and a beta-oncofetal antigen[238] which was demonstrated in 19 of 21 cervical carcinomas.

However, this beta-oncofetal antigen was also found in low concentrations in normal cervical tissue and in high concentrations in normal adult liver and kidney. Ghose et al. detected CEA in 16 of 16 squamous cell carcinoma of the cervix irrespective of whether the lesions were poorly or well differentiated.[239] The serum levels of CEA have been found to be elevated in a proportion of (but not all) cervical carcinoma patients.[237] The usefulness of beta hCG and AFP as markers of cervical cancer is controversial.[240] Herpes simplex virus 2 (HSV-2) antigens have been demonstrated in exfoliated cells of 100% of a group of patients with invasive and pre-invasive cervical carcinoma[241] and in 94% of another group with invasive cervical carcinomas.[242]

13. Neurogenic Tumors

a. Neuroblastomas

Neuroblastoma is a common childhood tumor that arises from primitive sympathetic neuroblasts[243] and presents diagnostic problems. Several MAB have been produced against human neuroblastomas. Examples include MAB P1-15 3/3 that reacts with neuroblastomas, glioblastomas, retinoblastomas, acute lymphoblastic leukemia cells (excluding leukemias of T-cell origin),[244] and MAB HSAN 1.2 that binds also to Wilm's tumors, Ewing's sarcoma, retinoblastoma cell lines, and fetal brain.[245] Similar cross-reactivity of other antineuroblastoma MAB with normal cells and tissues has been observed, e.g., with T-lymphocytes.[243] A MAB against the human Thy 1 antigen has also been found to react with neuroblastoma cells.[246] However, the Thy 1 antigen is expressed by various normal cell populations and tumors such as gliomas, myogenic sarcomas, teratomas, Wilm's tumors, and T-cell leukemias. Specificity analysis of MAB produced against tumors originating in neural crest-derived tissues has demonstrated extensive cross-reactivity among neuroblastomas, gliomas, melanomas,[247] and hematopoietic cell lines.[248]

Wilkstrand and Bigner[249] have recently listed MAB-defined human neuroectodermal tumor-associated antigens (HNTA). These include: (1) tissue-specific markers, many of which have been biochemically characterized (e.g., glial fibrillary acidic protein, S100, gangliosides, etc.); (2) shared nervous system-lymphoid antigens (e.g., Thy, HLA-DR, CALLA, etc.); (3) shared neuroectodermal and/or oncofetal antigens; and (4) putatively tumor-restricted antigens. The presence of markers under (1), (2), and (3) in one or more vital normal tissues (e.g., adult kidneys, brain, gastrointestinal tract, spleen, thymus, leukocytes, etc.) renders them unsuitable for tumor-targeting. Several MAB raised against melanomas have revealed specificity for gangliosides that are also expressed in gliomas and/or neuroblastomas and retinoblastomas, e.g., GD_2 (initially designated as OFA 1-2), GD_3, and GQ. Several anti-GD_2 MAB have also been produced after immunization of mice with human neuroblastoma cells.[250] In regard to normal tissues, GD_2 is mainly expressed by fetal brain. Although GD_3 is expressed by various normal tissues, e.g., normal brain, retina, and kidneys, MAB against melanoma-derived GD_3 binds poorly to normal tissues due either to a predominant intracellular location of the antigen or to minor chemical differences between melanoma-derived and normal brain-derived GD_3.[251] MAB P1 15 3/3[252] against a 30-kdalton glycoprotein is expressed in fetal brain and in glioma neuroblastoma and retinoblastoma cells as well as in B and null cell ALL and B-cell CLL. Antibodies against GD_2 and GD_3 and MAB P115 3/3 may have some potential for tumor-targeting. The specificity of those MAB that detect putatively restricted HNTA is yet to be established convincingly.

Most of the MAB against neuroblastomas bind to cell surface glycoproteins, many of which are probably differentiation antigens.[246] Recently, Cheung et al.[253] have produced a MAB against a cell surface glycolipid of somewhat restricted distribution, i.e., while the antigen is present on osteosarcoma and leukemic cells, it is absent from most Ewing's and Wilm's tumors. The MAB against this antigen reacted with more than 98% of cells in all surgically excised neuroblastoma specimens and were cytotoxic to 100% of neuroblastoma cells in the presence of complement. If the specificity of this antineuroblastoma MAB is confirmed, it appears to be very suitable for targeting chemotherapeutic agents in vivo. Some antineuroblastoma antibodies have been successfully used in treating autologous marrow,[254] localization of neuroblastomas in vivo,[255] and in treating neuroblastoma patients with chlorambucil- or daunorubicin-antibody conjugates.[256]

b. Glial Tumors

Appropriately absorbed xenogenic polyclonal antisera have detected common glioma antigens[249,257] and common astrocytoma antigens.[258,259] A rabbit antiglioma serum has detected a fetal brain antigen strongly expressed on gliomas and neurinomas.[260] A number of studies have demonstrated that sera from patients with brain tumors had a higher incidence of reactivity to tumor cells than did sera from normal donors.[261] MAB have been produced against human glioma-associated antigens by human-human,[262] human-mouse,[263] and mouse-mouse hybridomas. Although some of the MAB define antigens that appear to be shared and preferentially expressed by different histologic types of gliomas, none appear to be glioma or tumor specific. Vessels supplying intracranial tumors are not subject to the "blood-brain barrier".[249] Ghose et al. demonstrated that the $F(ab)_2$ moiety of a polyclonal antineuroblastoma antibody showed greater localization in mice than the parent IgG.[16,264] Furthermore, the delivery of MAB to brain could be increased fivefold by prior hyperosmolar perfusion of rats with 1.4 *M* mannitol or 1.6 *M* arabinose.[249] As already stated, many of the MAB produced against malignant melanomas, neuroblastomas, and hematopoietic cell lines also react with glial tumors.

14. Teratocarcinomas and Embryonal Carcinomas

Teratocarcinomas contain disorganized mixtures of adult, embryonic, and extraembryonic tissues that presumably arise by differentiation of pluripotent embryonal carcinoma cells.[265] Human embryonal carcinoma is believed to be of germ cell origin and multipotent in nature. Some embryonal carcinomas may be totally devoid of differentiation, whereas others may contain areas of choriocarcinomatous or yolk sac carcinoma differentiation, e.g., hCG and AFP, respectively. Several MAB have been raised against human teratocarcinomas, but none react exclusively with human embryonic carcinoma cells. For example, MAB 5.5.H., 8.7.D., and 13.7.A are directed against oncofetal antigens having extremely restricted in vivo tissue distribution.[266] The MAB described by Williams et al.[267] reacts with a 200-kdalton membrane glycoprotein on undifferentiated teratoma cells and cells lining human fetal intestine and bronchus. MAB TRA-1-60 and TRA-1-81 react with an antigen on undifferentiated embryonic carcinoma cells that is found also on other human tumor lines and several normal tissues.[268] A study using 3 MAB directed against high molecular weight glycoproteins of human teratocarcinomas and 165 human tumor lines and normal cells has confirmed the oncofetal nature of teratocarcinoma-associated antigens.[269] MAB against murine embryonic antigens such as SSEA-1, SSEA-3, and SSEA-4 react with moieties on the surface of human embryonic carcinoma cells or their differentiated derivatives even though there may be differences in the distribution of these antigens between the two species in regard to the differentiation status of tumor cell populations.[270] SSEA-1 is expressed by various normal and neoplastic human tissues.[271] Human embryonic carcinoma cells are SSEA-1 negative, although some components of germ cell tumors, e.g., yolk sac carcinoma and choriocarcinoma cells, express this antigen. The SSEA-1 epitope is a branched oligosaccharide that may occur as a part of a polylactosamide in the lacto-series of glycolipids. MAB against SSEA-3 react with human erythrocytes. The SSEA-3 epitope consists of the internal core of the oligosaccharide of globoside-7 (GL-7), whereas the SSEA-4 epitope consists of the terminal residues of this oligosaccharide. The globo-series of glycolipids contain the epitopes of the P blood group antigens.[272,273] Human teratocarcinomas produce very high levels of an alkaline phosphatase isozyme that is normally distributed in liver, bone, and kidney. After immunization of mice with a human teratocarcinoma line, two MAB against the liver/bone/kidney alkaline phosphatase isozyme have been obtained.[268] Ballou et al.[274] have successfully imaged xenografts of human teratocarcinoma in nude mice using a radioiodine-labeled IgM MAB against SSEA-1 and its $F(ab)_2$ moiety.[275]

III. PRODUCTION OF ANTI-TAA ANTIBODIES AND CRITERIA OF SUITABILITY FOR TARGETING

Details of the methods of production and purification of conventional polyclonal and monoclonal anti-TAA antibodies are beyond the scope of this review. The methods used by these authors for the production of polyclonal anti-TAA antibodies will be found in Ghose et al.[14] and other publications from the authors laboratory.[128,224,225,276] General methods for production of mouse hybridoma-derived MAB have been outlined[277,278] and methods for those against a given TAA are in the publications cited in Section II, e.g., colorectal carcinomas and melanomas,[155,247] antigens of human blood group systems,[74] lymphoma/leukemias,[120,121] ovarian carcinoma,[192] pancreatic carcinoma,[167] and mammary carcinoma.[173-175] The following comments are relevant to production of antibodies for targeting.

A. Rodent-Rodent Hybridoma Production

1. *Selection of Species and Strains*

Each species and strain of animal has a characteristic response pattern to a given antigen which must be taken into account. For example, some strains of rats do not respond to blood group A or B substances and are therefore suitable if one wishes to avoid the production of MAB against these antigens.[279] Whenever possible, more than one strain of mice should be used because the immune spleen cell donor and the immune response pattern of the species or strain of animal should be ascertained.

2. *Antigen to be Used for Immunization*

For the production of MAB against human TAA, the spleen cell donor can be immunized with: (1) tumor cell lines long maintained in culture; (2) freshly isolated tumor cells from patients; (3) membrane preparations or TAA-enriched fractions of cells from (1) and (2); and (4) serum free supernatants from the spent culture medium of tumor cells.[155,280] Tumor cells long maintained in culture tend to undergo antigenic modulation and lose certain antigens.[280] Their use yields MAB with low specificity, whereas use of freshly excised tumor tissue or TAA-enriched fractions derived therefrom yield MAB with more restricted specificity.[155,213] The use of pure TAA preparations yields highly specific MAB.[192,266] Methods of obtaining membrane preparations or TAA-enriched fractions from tumor cells have been outlined.[81,155,247,281] The use of formol-saline-fixed dehydrated tumor cells for obtaining a MAB that reacted with fixed tumor tissue has been reported.[267]

3. *Immunization Protocol*

For tumor localization in vivo, it is essential to have antibodies with high affinity. A single immunization of the spleen cell donor mouse is likely to yield low affinity antibodies so it may be advisable to use multiple injections, with or without adjuvants, for eliciting a secondary response.[155] The animals producing antibodies with the highest titer and affinity should be used for donating spleen cells for fusion. As most of the anti-TAA antibodies react in a limited way with one or more normal tissue components and no anti-TAA antibody reacts with a given histologic type of tumor, the reactivity with such tissues as renal glomeruli, bone marrow, etc. should be confirmed before use for targeting cytotoxic agents. Whenever possible, the tumor-specific localization of the carrier antibody should also be confirmed.

B. Human-Human and Human-Rodent MAB

The methodologies for the production of MAB of human origin are still in their infancy. Approaches that have been pursued[282] include the production of hybrids of lymphocytes from regional lymph nodes of cancer patients using rat or mouse myelomas,[172,283,284] human myelomas,[285] human B-cell lymphoma and lymphoblastoid lines,[282,286,287] or heteromyelomas.[282] An alternative approach for the production of human anti-TAA antibody-producing cell lines has involved the transformation of appropriately sensitized B-lymphocytes with Epstein-Barr virus.[282]

Human-rodent interspecies hybridomas are usually very unstable due to the selective loss of human chromosomes, and virus-transformed B-lymphocytes produce only small amounts of Ig and tend to cease antibody production after a variable period.[282,285] MAB of human origin are likely to be less immunogenic than xenogenic MAB for targeting anti-cancer agents in patients. However, the production of anti-idiotypic antibodies after repeated administrations of human MAB remains a possibility. Most human lymphocyte-derived MAB belong to the IgM class and have low affinity for TAA.[173]

C. Preparation of F(ab) and $F(ab)_2$ Fragments of Anti-TAA Antibodies

Several procedures for obtaining reactive fragments of Ig have been outlined elsewhere.[14,18] Lamoyi and Nisonoff[288] have demonstrated that mose hybridoma-derived MAB differ widely in their susceptibility to proteolytic digestion even within a given subclass. The products of digestion of MAB should be rigorously monitored using a two-dimensional gel electrophoresis along with appropriate marker proteins, and the reactivity of putative immunologically reactive fragments should be evaluated.[289,290]

D. Factors That Determine Tumor-Specific Localization of Antibodies

Factors that determine tumor-specific localization of systemically administered antibody preparations are outlined below. Many may be influenced by conjugation with agents (see below under methods of linkage).

1. Purity

The fraction of an antibody preparation that binds to available cell surface TAA is directly proportional to antibody purity.[289] Polyclonal sera are limited in their content of specific antibody even after affinity purification, which can yield preparations varying from 10%[291] to approximately 80% pure.[146] Furthermore, the extensive absorptions necessary for rendering polyclonal antibodies specific remove a substantial proportion of antibody molecules and leave behind contaminating antibodies that cross-react and bind to normal tissues. The antinormal tissue antibodies may contribute to systemic toxicity of conjugates.[292]

MAB, at least theoretically, consist of one clone of antibody molecules and therefore can overcome these difficulties. However, a source of inactive Ig molecules in MAB preparations is the secretion by the hybridoma of inappropriate hybrid Ig molecules that lack effective antibody-combining sites.[293] Also, in practice, when MAB are harvested from the ascites fluid of mice inoculated intraperitoneally with the antibody-producing clone, there is substantial contamination with mouse serum proteins that leak into ascites fluid. Isolation of MAB from ascites fluid presents in a limited way, the same problem as encountered during the purification of polyclonal antibodies. One method to overcome the problem is to grow the MAB-producing clone in vitro in serum-free medium[278] or in medium supplemented only with those components of fetal calf serum (FCS) that bind to protein A (if the MAB does not bind) and therefore can be removed by affinity purification with protein A. The problem of limited amounts of MAB that are usually obtainable from MAB-producing clones in culture (in contrast to harvesting MAB from ascites fluid) can now be overcome by methods for scaling up the production of MAB in vitro, e.g., either with the use of cytostat-growing clones inside hollow fibers or beads that are commercially available. Also, a commercial preparative HPLC purification system based on hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) is applicable to ascites fluid.

Ghose et al. compared the tumor-specific localization of ^{131}I-labeled polyclonal anti-human melanoma IgG, anti-human melanoma MAB 225.28S, and normal mouse IgG in xenografts of human melanoma in nude mice. The tumor-to-blood ratio of radioactivity was highest with the MAB and lowest with the normal mouse IgG.[139] However several groups of investigators did not find any difference between monoclonal and polyclonal anti-CEA antibodies in regard to their localization in tumors and clearance from serum.[93] Stuhlmiller et al.[294] studied tumor localization of ^{131}I-labeled anti-human-melanoma antibodies of monkey, human (i.e., serum of patients immunized with allogeneic melanoma cells), and murine hybridoma origin in human melanoma xenografted nude mice. Compared with different control nonspecific IgG preparations, each specific antibody showed selective localization in tumor tissue. The ratio of tu-

mor-to-normal tissue localization was highest with the monkey antibody and lowest with the murine MAB.

2. Affinity for TAA

At a given antigen concentration, the affinity constant of an antibody determines the amount that can bind to target cells.[289] In turn, the amount of an antibody-linked drug (i.e., MTX) that is endocytosed is correlated with the amounts of carrier antibodies observed to bind at equilibrium at 0°C and the titer of the binding.[295] Antibodies that have low affinity, either intrinsically or as a result of the method of conjugation, are not likely to be effective for targeting. Even the fraction that binds is rapidly lost from the tumor.[296] Kennel et al.[289] have calculated that antibodies with binding constants of less than 108 M^{-1} are not likely to be useful for drug targeting or tumor imaging, assuming a value of 10^6 antigenic sites per cell. MAB are notorious for their low affinity. However, repeated immunization of splenic cell donor mice may yield hybridomas that produce high affinity antibodies.

3. Specificity

As already discussed, TAA that are exclusively tumor specific have yet to be found. However, it is the difference in the amount of the TAA (and the difference in their affinity for the antibody if any) in the target tumor and surrounding normal tissues that will be critical for drug-targeting. For example, oncofetal antigens that are not expressed or are expressed only in very limited amounts in normal adult tissues are likely to be adequate for drug-targeting. On the other hand, several anti-CEA MAB failed to localize in tumors and gave rise to systemic complications because of their cross-reactivity with CEA epitopes on normal cells.[92]

4. Concentration and Availabilty of TAA

The amount of antibody that will bind to tumor cells for a given MAB-TAA system will vary with the concentration of TAA sites.[289] The number of sites available on cells of experimental tumors has been calculated to be 2.5×10^4 to 7×10^6 per cell.[12,289] Results from our laboratory show that TAA sites on human melanoma cell lines fall within this range. According to Kennel et al.,[289] the TAA concentration in such a system is likely to be in the range of 10^6 to 10^9 M, but rarely above. The conditions that will determine the interaction between a fluid-phase drug carrying antibody and TAA in solid tumors are complex and include the following: (1) accessibility of TAA to conjugates in intravascular and extravascular fluids; (2) rate of diffusion of conjugates in the extravascular compartment; and (3) susceptibility of TAA to modulation and the rate of regeneration of TAA sites after endocytosis. TAA-antibody combinations in which the TAA modulates and becomes cryptic upon exposure to the antibody are obviously unsuitable for drug-targeting. Endocytosis of the carrier antibody is essential, if the cytotoxic agent is not surface active and its target molecules are intracellular. Comparison of the rate of endocytosis of three different MTX-linked antimelanoma antibodies has indicated differences in the rate of regeneration and recycling of TAA sites. The binding sites for MAB 225.28S regenerated faster and were more efficient for internalization of antibody-linked MTX than the binding sites of two polyclonal antimelanoma antibodies.[295] Some cell-surface TAA do not undergo endocytosis even after antibody-induced capping. For example, Ghose et al. observed capping and endocytosis of antibody-bound TAA on EL4 cells[297] but not with TAA on H6 hepatoma cells.[292]

5. Influence of Secreted or Shed Antigens(s) in the Millieu of Tumor Cells

Many TAA are either actively secreted (e.g., hormones, PAP, AFP, etc.) or shed

(e.g., MAA) in the extracellular fluid. Some TAA may also be released by dead or dying tumor cells. The concentration of such extracellular TAA is likely to be the highest in the immediate vicinity of tumor cells, especially in those areas where vascular flow is sluggish and lymphatic drainage is inadequate. The presence of extracellular antigen(s) in solid tumors is unlikely to be a serious impediment for imaging or therapy with antibody-linked radionuclides. In fact, the presence of large amounts of localized free antigens may increase the number of binding sites per unit volume of tumor tissue and thus lead to increased tumor localization of the agent. However, the presence of extracellular free antigen may interfere with the effectiveness of those conjugates of cytotoxic agents that need internalization for cytotoxic effect. On the other hand, dissociation of an active drug from the extracellular immune complexes may produce a local depot effect, i.e., a slow prolonged action of the agent at the tumor site. This will be unlikely to happen with immunotoxins constructed with only the A chain of the toxin molecule unless the A chain also contains the part of the B fragment (in a cryptic form) that facilitates internalization. Local extracellular accumulation of TAA-immunotoxin complexes may then result in tumor inhibition.

Apart from concentration, the affinity between free TAA and carrier antibodies is also an important factor in determining the effectiveness of antibody-linked agents. Interactions between antibodies and cell surface TAA can be regarded as a typical fluid phase mono- or bivalent reaction.[289,293] At least some of the membrane-bound TAA may behave atypically because of adjacent molecules (e.g., steric hindrance of glycolipid TAA by other membrane-located glycolipids,[298] membrane fluidity, and the relatively slow diffusion constants of membrane-bound antigens that may prevent the proper orientation of the epitope for binding with the antibody). Any advantage in affinity of extracellular TAA over cell surface-bound TAA will detrimentally affect the binding of carrier antibodies to tumor cell surface.

6. Interference by Host Antibodies and Immune Complexes

Although oncofetal antigens are usually not antigenic in the tumor host, other TAA (e.g., cell surface-associated neoglycoconjugates or blood group precsursors) may provoke a response in tumor hosts. Autologous anti-TAA antibodies may compete with drug-linked xenoantibodies for binding sites on the tumor cell surface. Relative concentrations of the two antibodies in the milieu of tumor cells and their affinity constants will be among the factors determining the amounts of the two antibodies that will bind to tumor cells. Autologous antibodies may react with circulating TAA to produce immune complexes which are likely to be in the zone of antigen excess at the tumor site. These complexes in antigen excess may also neutralize administered antibody conjugates. If the carrier antibody possesses a higher affinity for the TAA than the autologous antibody, then the carrier antibody is likely to displace the autologous antibody from complexes, causing further "neutralization" of the conjugate. Autologous antibody displaced from immune complexes will compete with the unbound antibody conjugates for TAA on the tumor cell surface. If the conjugated antibody happens to have a higher affinity for free TAA (or TAA in the immune complex) than for cell-bound TAA, there is likely to be substantial reduction in the binding of the conjugate to tumor cells. Furthermore, there will be additional loss of binding of the conjugate to tumor cells if the autologous antibody happens to have higher affinity for the cell-bound TAA than the carrier antibody.

7. Size of the Conjugated Antibodies

The use of F(ab) and $F(ab')_2$ fragments, instead of the entire Ig molecule, is likely to: (1) augment transcapillary passage and diffusion in extracellular space; (2) enable the carrier moiety to cross the blood brain barrier and thus make the carrier suitable

for the imaging or therapy of tumors in the central nervous system;[16,264] (3) reduce the antigenicity and susceptibility to phagocytosis; and (4) lead to faster clearance of the labeled moieties. Indeed, F(ab) and $F(ab')_2$ moieties of anti-TAA antibodies are cleared from the circulation at a much faster rate.[17] Rapid clearance of labeled carriers that are not tumor bound reduces background binding to normal tissues and improves the ratio of tumor to normal tissue localization of the carrier. This is of help in tumor imaging because a high tumor-to-background ratio is likely to allow resolution of smaller tumors and/or tumors with low uptake of targeted radionuclides. However, the therapeutic effectiveness of a given dose of conjugates is decreased because the proportion of the total administered amount of the $F(ab)_2$ moiety that localizes in a tumor is much less than that of the parent IgG.[17] The rapid clearance of labeled $F(ab')_2$ and F(ab) fragments from blood is likely to shift the equilibrium of binding, leading to the dissociation of the bound antibody away from the tumor. We have observed that MTX linked to the $F(ab')_2$ moiety of an anti-EL4 lymphoma IgG was a less effective tumor inhibitor than equivalent amounts of MTX linked to the parent IgG.[299]

Since an antibody-cell-surface TAA interaction can be regarded as a monovalent reaction,[289] it is not surprising that IgG antibodies and their F(ab) and $F(ab')_2$ fragments have nearly identical affinity constants.[293] However, monovalent carriers like the F(ab) fragment will be less susceptible to endocytosis or shedding after binding to the tumor cell surface because monovalent fragments do not cap. Although lack of shedding or endocytosis may improve the imaging quality of antibody-linked radionuclides and the therapeutic effectiveness of conjugated cell-surface active agents, it will render targeting ineffective for agents that interact with intracellular targets.

8. *Dosage of Administered Conjugated Antibodies*

If a cell surface TAA is freely exposed to circulating blood (e.g., as with leukemic cells), the amount of a conjugate that would bind to tumor cells will depend upon the concentration of the labeled antibody, the number of TAA sites available for binding, the affinity of the antibody, the amount of competing antibody (either in the conjugate preparation, or in the form of autologous anti-TAA antibodies), and circulating antigen. However, with "solid" tumors, additional factors will be the fraction of the cardiac output that reaches a tumor (usually small), the extent of the tumor bed to be supplied by the blood delivered, the capacity of the conjugate to cross the capillary wall, its rate of diffusion and stability in the extravascular compartment (especially in the acidic milieu),[277] and the high content of proteolytic enzymes that are usual in solid tumors. It has been recently reported that in melanoma patients given ^{111}In-labeled MAB 96.5 (directed against the P97 melanoma antigen) the number of tumor sites imaged increased with the amount of antibody administered.[140] It is possible that increasing the total antibody dose will saturate nontarget sites and thus further increase the concentration of the MAB in the milieu of tumor.

9. *Pharmacokinetics*

Even very mild treatment of antibodies during conjugation may alter their pharmacokinetics in vivo and reduce their circulating half-life as a result of rapid elimination of denatured antibodies.[300] Also, substantial uptake and catabolism of denatured conjugates by phagocytic cells could contribute to nonspecific localization. Another cause of nonspecific localization and rapid elimination of antibody agents is the presence of TAA or antigenically related materials in the circulation. There is usually an elevation in the serum level of many of the TAA and tissue-specific antigens (e.g., prostate-specific antigens) in cancer patients.

E. The Rationale and Limitations of Cancer Therapy with Antibody-Targeted Agents

These have been discussed before.[12,15] The rationale for the use of MAB as carriers of cytotoxic drugs in treatment is based on the demonstration that intravenously administered anti-TAA antibody shows target-selective in vivo localization.

1. Tumor Localization of Anti-TAA Antibodies

The evidence for tumor-specific localization of intravenously administered antibodies and the factors that determine the amount that reaches the tumor and its duration of sojourn will be briefly summarized. In evaluating the tumor-specific localization of anti-TAA antibodies, it should be remembered that considerable accretion of intravascularly administered macromolecules can occur in a tumor as a result of increased tumor vasculature, increased permeability of newly formed vessels, and the lack of lymphatics in tumors.[139,225] A variety of appropriately radiolabeled macromolecular carriers including serum albumin have successfully imaged a sizable proportion of human tumors due to intratumoral vascular and hemodynamic changes (i.e., sluggish flow of blood, increased blood volume, and the increased permeability of vessels inside tumors). Therefore, to demonstrate tumor-specific localization of anti-TAA antibodies, it is not only necessary to show higher localization of the labeled antibody in tumors compared to its localization in normal tissues, but also higher intratumoral localization of the labeled antibody compared to an immunologically irrelevant antibody of the same class and subclass. In one approach to establish tumor-specific localization of anti-TAA antibodies, anti-TAA and nonspecific Ig labeled with different radioiodine isotopes have been used, i.e., the so-called "paired label method".[300] Using these stringent criteria, the tumor-specific localization of both polyclonal and monoclonal anti-TAA antibodies and their immunologically reactive fragments have been established in experimental tumors including leukemia/lymphoma,[225,274,301-303] in human tumor xenografts,[139,304,305] and in cancer patients.[142,143,147,225] For example, using an ^{131}I-labeled MAB against melanoma TAA, Ghose et al.[139] and others[306] have achieved a several-fold increase in localization in tumors compared to other tissues. Specific tumor localization of MTX linked to anti-EL4-IgG (AELG) has been demonstrated by Ghose et al.[307]

For effective eradication of tumor cells by antibody-linked cytotoxic agents, it is essential that a sizable proportion of the administered antibody localizes and remains in the target tumor. However, experiments in the authors' laboratory showed that only approximately 0.55 to 0.65% of the administered activity of a ^{131}I-labeled antimelanoma MAB localized in human melanoma xenografts in nude mice at 48 hr; at 126 hr the proportion was still less, i.e., 0.27 to 0.56%.[17] With purified ^{131}I-labeled goat anti-CEA IgG, Mach et al.[147] have observed that while the concentration of the antibody in the tumor could reach ninefold higher than in the liver only 0.05 to 0.2% of the injected radioactivity was found in the resected tumors 3 to 8 days after injection. However in other tumors, uptake of MAB-bound radionuclides was much higher, e.g., 5 to 10% of the administered dose in the tumorous spleens of erythroleukemic mice,[308,309] 6.5% of the administered dose per gram in s.c. transplants of Thy 1.1 positive mouse lymphoma,[310] up to 20% of the total body activity in human tumor-xenograft-bearing nude mice,[311] and 17.2% of the administered dose in human melanomas (0.04% per gram of tumor tissue) after injection with the ^{131}I-labeled F(ab) fragment of anti-p97 MAB.[143] The interval between injection and the observed maximum was as short as 6 hr in erythroleukemic mice.[309] In Thy 1.1 positive s.c. transplants of a mouse lymphoma, the level of antibody rose in tumors over the first 24 hr and was maintained at this level for approximately 24 hr. The level declined exponentially thereafter ($t_{1/2}$ 104 hr), with a mean of 3% per gram remaining at 8 days. In contrast, only 1.5% per gram of a control antibody was present at 24 hr and remained in the tumor at that level over

8 days.[310] It is possible that the proportion of antibody that localizes in tumors can be increased by using a cocktail of antibodies against different TAA or different epitopes of the same TAA. DeLand and Goldenberg[93] have reported that a mixture of three MAB against GW39 did not produce a greater concentration of activity in the tumor than administration of a single MAB. However, it was not stated whether these three MAB were directed against the same epitope.

High affinity antibodies are likely to remain localized in tumors longer than antibodies with low affinity. It is therefore encouraging that the $F(ab')_2$ and F(ab) fragments of anti-TAA antibodies retain the affinity of the parent IgG molecule.[93,142] Additional factors that are likely to influence the proportion of antibody that localizes in tumors and the kinetics of localization include: (1) tumor size and weight (a direct relationship between tumor size and uptake has been attributed to probable nonspecific trapping);[311] (2) tumor vascularity; and (3) catabolism of the conjugate at the tumor site and removal of catabolized fragments. Catabolism of tumor-bound antibody has been observed between 6 to 24 hr of injection and has been found to be independent of Ig class and reactive fragment used.[309] Tumor-bound antibody may be released as a result of shedding of the bound antigen, endocytosis followed by intracellular catabolism, proteolytic activity on and around tumor cells, and normal turnover of TAA and membrane-associated glycoproteins. Major limitations of antibody-targeted drugs in the treatment of cancer include the following.

2. Circulating Antigens, Immune Complexes, and Blocking

In two studies on MAB therapy of leukemia, circulating antigen effectively blocked binding of the MAB to target cells in vivo.[312,313] However, blocking was overcome after repeated infusions in the study of Miller et al.[312] and not observed in several other serotherapy trials.[118,314] In melanoma patients with elevated serum levels of P97 antigen, lesions could not be imaged with ^{111}In-labeled MAB against the P97 antigen.[140] There are other reports showing that high serum levels of TAA such as CEA or hCG do not appreciably interfere with tumor localization of administered antibodies.[93,146] So far, the presence of a blocking factor does not appear to be predictable. However, the nature and cell surface distribution of an antigen may be a fair indicator of the possible presence of TAA in the circulation (see below). The amount of circulatory antigen appears to be related to tumor cell burden, and therefore circulatory blocking factors may not be a major factor if the tumor burden is reduced first by other modalities of therapy. Furthermore, elicitation of antibody responses to the carrier xenoantibody[141] and/or linked agents, i.e., drugs and protein toxins,[315] may limit the usefulness of conjugates.

3. Antigenic Modulation

Several studies have revealed antigenic modulation in target tumor cells both in vitro and in vivo.[118,316] However, these cells were able to reexpress the antigen when antibody was no longer present. Furthermore, antigenic modulation occurred only with certain cell surface antigens such as CALLA and surface Ig, but not with others, such as Ia and histocompatibility antigens. There appear to be several different mechanisms of antigenic modulation.[317] These include rearrangement of the antigen in the cell membrane (e.g., TL antigen), shedding of surface antigens (e.g., some MAA), and internalization of antigen, either alone[186] or antibody bound. Examples of the latter are the CALLA and EL4 mouse lymphoma antigens.[297] Proper scheduling of antibody infusions to allow reexpression of the antigen should overcome this problem. It may also be possible to select a MAB against an antigen that does not modulate.[118] Antigens such as CALLA or the prostatic P54 antigen[186] that internalize along with bound an-

tibody may be ideal targets for MAB-linked agents if the target molecules of the conjugate are intracellular.

4. Tumor Cells in Immunological Sanctuaries

Tumor cells in immunological sanctuaries such as the central nervous system may not be accessible to infused antibody conjugates. However, Ghose et al. demonstrated that the $F(ab')_2$ fragment of antitumor IgG can cross the blood brain barrier and selectively localize in intracerebral tumors.[16,264]

5. Antigenic Heterogeneity of Tumor Cells

Most, if not all, human tumors investigated have proven to be antigenically heterogeneous, i.e., 100% of any given histologic type of tumor does not react with a given anti-TAA antibody. Within a given tumor, cells show a wide variation in their reactivity to a given anti-TAA antibody and usually a proportion of cells does not have detectable TAA. Foulds[318] reported morphological heterogeneity in different areas of a single mammary tumor. More recently, the results of the reaction of 39 different human mammary carcinomas with 4 anti-TAA MAB have been discussed by Schlom et al.[173] Ten antigenic phenotypes emerged, ranging from those that express all four antigens to those that express none. There were also wide variations in the expression of TAA by different cells within a given tumor. One area of a tumor contained cells that expressed a particular TAA but in another area of the same tumor, cells lacked this TAA. TAA positive cells were found directly adjacent to TAA negative cells even in a given area. The TAA distribution in a positive cell population varied, i.e., TAA could be found focally in the cytoplasm, diffusely throughout the cytoplasm, or in the luminal borders of cells. Similar antigenic heterogeneity has been observed in most human cancers investigated, e.g., lung cancers,[212,319] melanoma,[320,321] prostatic carcinoma,[181] and others. Heterogeneity of tumor cells has been observed even after cloning.[280] Some MAB react with a proportion of cells in the primary tumor but with none in one or more metastatic lesions. For antibody-linked agents to be therapeutically effective, the reactivity of tumor cells with the carrier antibody should be closely monitored and, in certain cases, a cocktail of antibodies that react with all cells in a given tumor may be useful. It has been claimed[173] that biologic response modifiers such as interferon can induce the expression of TAA in antigen-negative tumor cell populations. Another approach to overcome antigenic heterogeneity of tumor cells, especially "patch-work" heterogeneity, is the use of appropriate radionuclides (instead of chemotherapeutic agents and protein toxins) so that the tumoricidal dose of radiation can extend up to several cell diameters.[15]

6. Relative Lack of Tumor-Specificity of Anti-TAA Antibodies

The lack of absolute tumor specificity of available MAB also remains a formidable problem. For example, CALLA is expressed not only by non-T-cell ALL (80% of patients), blast cells in CML crisis (40% of patients), and a variety of lymphomas, but also by a small proportion of normal bone marrow cells. More pertinently, in most of the clinical studies on the serotherapy of T-cell ALL, T-cell lymphoma, and B-cell CLL, the MAB used not only reacted with tumor cells but also with T-cells in peripheral blood, spleen, lymph nodes, and thymus.[118] Effective tumor suppression also led to immunosuppression. Furthermore, the presence of large numbers of non-neoplastic antigen-bearing cells would interfere with the binding of the passively administered antisera to tumor cells and would require infusions of larger amounts of xenoglobulins. Unfortunately, MAB that appear to be more specific for tumor cells are directed against antigens that are only expressed in the tumors of a small proportion of patients (e.g., MAB 89 against a human lymphoma-associated antigen,[322] MAB 4C7 and 3C2

against ovarian carcinomas,[323] MAB against non-SCLC lung tumors,[324] a MAB against colorectal carcinomas,[155] and MAB against breast carcinomas[172]). The idiotypic configuration of the B-cell surface Ig furnishes what is, at present, probably the most specific TAA. With a cytotoxic agent linked to an anti-idiotypic antibody, the only normal cell population that would be destroyed would be those B-cells that have the same idiotype as the tumors. Antibodies directed against the idiotype configuration of T-cell receptors, when available, are likely to have the same degree of specificity. Although inhibition (or eradication) of non-vital normal cell populations (e.g., prostatic tissue and endocrine and exocrine glands) may not always be a serious limitation, MAB of greater tumor specificity should be sought.

7. Emergence of Resistant Tumor Cell Populations

It is possible that tumor cell populations that lack the targeted TAA or are resistant to the cytotoxic agent will emerge after repeated administrations of antibody-linked agents. Ghose et al.[128] and others[118,316] have observed the emergence of antigen-negative tumor cell populations either as a result of modulation or of immunoselection of antigen-negative tumor cell variants after prolonged administration of antibody, either alone (leukemia patients) or linked to chemotherapeutic agents (melanoma patients). Use of carrier antibodies directed against different TAA and the use of alternate chemotherapeutic agents that exert a cytotoxic effect on the resistant tumor cell population may overcome this problem. Fortunately, methods are now available for binding different classes of cancer chemotherapeutic agents and a variety of protein toxins for the production of therapeutically effective conjugates.[14,18]

8. Lack of Vascularity in Solid Tumors

It is unlikely that systemically administered anti-TAA antibodies would reach avascular and necrotic areas in "solid" tumors. In fact, Ghose et al. failed to observe any localization of the labeled antibody in necrotic areas in tumors or in necrotic tumors in patients with primary or metastatic renal carcinoma given ^{131}I-labeled polyclonal antirenal carcinoma IgG.[225] It should be emphasized that surgery and/or radiation are likely to remain the effective and preferred methods for the treatment of localized tumor masses. At present, chemotherapy alone is used in the treatment of disseminated tumor cells. It is hoped that the use of an antibody-linked cytotoxic agent will increase the therapeutic index of that agent, i.e., render it more tumor selective. Furthermore, the additive and occasionally synergistic antitumor actions of anti-TAA antibodies and chemotherapeutic agents may add to cytocidal action.[12] The cytocidal action of most chemotherapeutic agents follows first-order kinetics (rendering the probability of the eradication of the last tumor cell low), whereas the action of anti-TAA antibodies alone or agent-linked antibodies follow zero-order kinetics. Therefore, antibody-linked cytotoxic agents are likely to be more effective in eradicating circulating tumor cells and microemboli than unconjugated agents.[12] Thus, this modality of therapy is likely to be most effective in the eradication of residual disseminated cells, micrometastases, and leukemias after reduction of tumor mass by other modalities of therapy.[29] Furthermore, large tumor burdens are also likely to be associated with elevated levels of circulating TAA that would "neutralize" antibody-linked agents.

9. Toxicity and Detrimental Effects of Antibody-Linked Agents

Untoward effects of agent-antibody conjugates may be due to either components, as well as to contaminants such as pyrogen. Adoption of stringent aseptic procedures and routine testing of preparations for pyrogen virtually abolished the risk of pyrogen-induced reactions.[225,276] Adverse effects due to antibodies include the following. There may be reactivity of the carrier antibody with nontarget cells due to a lack of absolute

tumor specificity of the carrier antibody, e.g., the systemic toxicity of an anti-CEA antibody that cross-reacted with circulating granulocytes and erythrocytes,[92] or the suppression of immunological reactivity as a result of the cross-reactivity of antilymphocytic leukemia antibody with immunocompetent cells.[118] Foreign Ig, as well as the ligands, are likely to be immunogenic and, after repeated administrations, may provoke an immediate hypersensitivity reaction[276] or immune complex-mediated reactions due to the production of precipitating antibodies against xenoglobulins. Such antibodies are also likely to interfere with the tumor-specific localization of conjugates and divert conjugate-containing immune complexes to organs rich in reticuloendothelial cells such as the liver and spleen.[92] However, after removal of aggregated Ig from chlorambucil-antimelanoma IgG conjugates, Ghose et al. failed to detect any precipitating antibody in patients given repeated injections of chlorambucil-polyclonal IgG.[276] Removal of Ig aggregates and initial high doses of anti-TAA Ig may induce tolerance instead of provoking a humoral response. Administration of murine hybridoma-derived MAB to patients with lymphoproliferative disorders[118,312,325-327] or mammary carcinoma[173] had very few ill effects. However, occasional adverse effects after infusion of MAB have been reported.[317] In the trial conducted by Miller et al.[328] on the effect of MAB anti-Leu 1 in T-cell lymphoma, antibodies to mouse Ig appeared in four of seven patients and contributed to the loss of effect of the antibody in three patients. Dillman et al.[329] attributed the lack of effectiveness of MAB T101 in two of four cutaneous T-lymphoma patients to the development of antimouse Ig antibodies. Sears et al.[330] could detect human antimouse Ig antibodies in 9 of 18 gastrointestinal carcinoma patients receiving MAB 1083-17-1A, but no adverse effects were noted.[312,325-327,331] In an extensive study, Schroff et al.[141] assayed the serum level of antimouse Ig in CLL, cutaneous T-cell lymphoma, and malignant melanoma patients. Elevated serum levels of antimouse Ig were seen in 0 of 11 of the leukemic patients and 3 of 9 of the melanoma patients. In one melanoma patient, the antibody appeared to be anti-idiotypic. No clinical toxicity was observed.

Immunologically reactive fragments of anti-TAA antibodies from which the Fc moiety has been removed may be less immunogenic. The availability of anti-TAA MAB of human origin is also likely to reduce immunogenicity. It is possible that recombinant DNA technology will provide human MAB after the isolation and linkage of genes coding for the antigen-binding region and the rest of the Ig molecule.[173] In addition to routine assay of serum for the detection of antibody against xenoglobulins, patients who are being given repeated injections of antibody-linked agents should be monitored by skin testing for their reactivity to conjugates during the administration of agents.[276]

Cytotoxic agents in conjugates may also cause adverse effects. After the linkage of low molecular weight cancer chemotherapeutic agents to Ig, there are likely to be changes in the toxicity and pharmacokinetic properties of the linked agent. For example, the rate of clearance of MTX from the circulation is considerably reduced with concomitant increase in its toxicity when the drug is administered as an IgG-conjugate.[307] On the other hand, the toxicity of several other cancer chemotherapeutic agents is reduced after linkage to macromolecular carriers, e.g., adriamycin and Trenimon.[13] However, cleavage of such agents from the carrier either in circulation or after intracellular catabolism may enhance the toxicity of the preparation. This is especially pertinent when conjugates containing both the A and B chains of protein toxins are used. Even when conjugates contain only the A chain, phagocytosis or pinocytosis of conjugates by non-target cells can contribute to systemic toxicity and lethality.[332]

IV. METHODS OF LINKAGE OF CYTOTOXIC AGENTS TO ANTIBODIES

Reviews referred to in the introduction include descriptions of binding methods.[13,14,18] Here, a summary of general principles highlighting major methods along with selected examples will be given.

A. General Principles

1. Preservation of Antibody Activity

Preservation of the activity and affinity of the carrier antibody is essential but may be difficult to achieve in practice. A linkage procedure that takes place under mild conditions is desirable since it appears that Ig, especially MAB, vary widely in their susceptibility to denaturing conditions. Even very slight denaturation of antibodies during labeling may alter their pharmacokinetics in vivo, e.g., the half-life of circulating antibodies has been observed to be considerably shortened after labeling with ^{131}I.[300] The presence of the bound agent may also alter the conformation of the combining site, especially if the agent is distinctly hydrophobic or possesses multiple charged groups. Agents, particularly proteins, may produce steric hindrance of antigen binding. Substitution may occur in the antigen-combining site of the Ig and prevent access by the antigen if the reactive functional group being used for the conjugation reaction is present in that site as well as in other regions of the Ig. These effects are likely to become more pronounced as the incorporation ratio increases. For example, when agents such as chlorambucil, MTX, adriamycin, daunorubicin, etc. are directly linked to Ig, loss of antibody activity can be substantial when the molar incorporation ratio of drug to antibody exceeds 10.[14] According to Pressman,[300] radioiodination at the level of less than 2 atoms of iodine per IgG molecule did not affect the activity of tumor-localizing antibodies, but localizing activity was reduced to 30% of the original when 19 atoms of iodine were present. In comparing the use of reactive fragments instead of the intact Ig molecule, Ghose et al. observed that that $F(ab')_2$ moiety of a rabbit anti-BSA IgG lost antibody activity at lower levels of incorporation of MTX compared to the parent IgG.[299] Measures may be adopted to protect the antigen-binding sites. An example is immobilization of the antibody on antigen-containing matrices during conjugation procedures.[300] The choice of linkage groups in the Ig (e.g., carbohydrates or SH groups) that are likely to be absent from the antigen-binding site may also promote retention of antibody activity.[14,18]

2. Preservation of the Activity of the Cytotoxic Agent

The activity of a cytotoxic agent in conjugate form must be considered in relation to the in vivo disposition of the conjugate. Either the activity must be preserved in the intact conjugate or provision must be made for the release of an active cytotoxic agent at the target site. If the conjugated agent can ultimately be released at the target site in an active form (but not necessarily its original form), there is no restriction on its interim chemical manipulation associated with the linkage reaction. In this context, the linkage reaction must either be reversible or susceptible to metabolic action which, in effect, generates an active agent. If the agent is to act on its target site in conjugated form, either intact or as a metabolic fragment, then groups in the agent essential for its action must not be used for linkage and must not be sterically hindered by other groups in the molecule involved in the linking bond. Also, retention of activity is more likely if a nonessential group is linked via a spacer arm to the Ig since steric considerations may be paramount.

3. Pharmacokinetic Considerations

A conjugate must be stable in transit via the circulation and extracellular space to target sites. The linkage reaction must neither be too readily reversible nor inappropriately susceptible to enzymatic disruption or the agent is liable to be released from the carrier prior to reaching the target cell. Aside from this consideration, in many cases chemical linkage designed to appropriately release the bound agent in vivo would constitute the ideal method. However, restrictions on the functional groups available in both agent and Ig limit such design.

High molar incorporation of drugs into Ig with the use of spacers and intermediaries or the linkage of large protein toxin molecules to antibodies may substantially alter the size and charge of the carrier antibody. This can impede its transcapillary passage and diffusion in tissue space and ultimately alter its pharmacokinetics. Furthermore, large and/or negatively charged conjugates are likely to be rapidly cleared from the circulation by phagocytic cells of the reticuloendothelial system and thus diverted from the target tumor tissue.

4. Functional Groups Used for Binding

Binding methods depend on groups that are present or can be introduced into the agent and Ig and the number of reactive groups in Ig limits the number of molecules of agent that can be attached. Reactive groups in Ig potentially utilizable for linkage occur in the side chains of several of the 20 amino acids as well as in the carbohydrate moieties.[14,18] They include aliphatic carboxyl, amino, disulfide, and hydroxyl groups; imidazole and phenolic rings; aromatic hydroxyl groups; and vicinal diols. The most widely used thus far have been carboxyl and amino groups. Introduction of reactive functional groups into Ig is exemplified by a number of variations in approach to linking through sulfur-containing bridge moieties based on the use of heterobifunctional reagents.[14,18] These have been applied most frequently to conjugating protein agents, e.g., toxins, so they will be discussed below under that heading. The production of F(ab)-SH by reductive splitting of IgG also represents, in effect, the incorporation of an -SH group.

Functional groups in agents also require careful consideration.[14,18] Those in protein agents are essentially the same as outlined for Ig, so here low molecular weight chemotherapeutic agents will be considered. The carboxyl group has often been used because it allows mild coupling reactions. If more than one is present in a drug, e.g., MTX, there is the possibility of activation of additional groups leading to cross-linking. For this reason, it may be desirable to limit the molar ratio of reagent to drug during the activation stage or utilize a regiospecific method. Other groups of importance, because of convenient linkage chemistry, include the amino group, the hydroxyl group, and vicinal dihydroxyls. Incorporation of reactive functional groups into drugs may also be feasible. However, the structural chemistry of drugs and their derivatives is outside the scope of this review, so the literature on individual drugs should be consulted; only a few examples pertinent to conjugate design are included here. Carboxyl groups can be introduced by reaction of an existing amino group with an anhydride. An interesting example of this is the use of *cis*-aconitic anhydride to produce a carboxy derivative which is susceptible to hydrolytic decomposition under mildly acid conditions, such as exist in lysosomes.[333] Succinic anhydride forms an analogous, more stable derivative.[334] Introducing a carboxyl group in this way also has the effect of introducing a potential spacer and this aspect is discussed below. A derivative of daunorubicin can be obtained in which the methylketone side chain is modified to the corresponding 14-bromomethyl ketone. The activated halogen atom alpha to the carbonyl group is capable of reaction with protein amino (and carboxyl) groups. Zunino et al.[335] prepared stable covalent conjugates with a number of proteins by incubating

14-bromodaunorubicin with the protein at pH 8.5. Molar incorporation ratios ranged from 0.1 (lysozyme) to 3.7 (BSA) and 8.5 (Ig).

B. Outline of Binding Methods

Important linkages utilized for conjugation include amides, disulfide bridges, and Schiff base-derived groups. Stabilities in vivo can range widely and are likely to be influenced strongly by steric factors arising from the juxtaposition of agent and Ig moieties. Representatives at the low to moderate end of the scale include esters and amides that are susceptible to lysosomal hydrolases. Disulfide bridges are likely to be susceptible to reductive splitting, whereas the thioether bond would be expected to be more stable. The behavior of conjugates based on linkage groups such as aziridyl amides, hydrazones, and Schiff bases will be influenced by their chemical lability. This could be of value as long as the agent was not released prior to reaching the target tissue. An aziridyl amide is relatively stable at the pH of blood but is hydrolyzed at pH 4 with a half-life of a few hours so breakdown in lysosomes should be promoted.[336] In this context, various lysosomotropic conjugates[337-339] have been designed and some are discussed below. When spacers or intermediaries are introduced, there can be several linkage bonds between the agent and the Ig, and the properties of these multiple groupings may be important in the manifestation of a cytotoxic action. A dramatic example is the difference in cytotoxicity between the *cis* aconityl and maleyl derivatives of daunorubicin. The most labile link will govern in vivo splitting of the conjugate into a "protein fragment" and an "agent fragment".

1. *Noncovalent Binding*

Noncovalent binding directly between agent and antibody has not been generally applicable. Parameters involved have been discussed by Edwards[340] and by Ghose et al.[14] One noncovalent-binding system that has potentially more general applicability to conjugate synthesis is avidin-biotin (see Ghose et al.[14]). The methodology entails linking the agent to avidin and the Ig to biotin, or vice versa, followed by combination of the two binary conjugates. Successful conjugation depends on not blocking either the avidin or the Ig-binding sites. Although the binding between avidin and biotin is noncovalent, the dissociation constant is of the order of 10^{-15}.[341] This is indicative of very high stability, and a combination of low pH and denaturing agents or proteolysis would be required to split a conjugated agent. The analog, 2-imminobiotin, forms a strong interaction at high pH values, but dissociation is easier at low pH where it becomes positively charged.[341]

The avidin-biotin interaction has been applied recently to toxin-antibody conjugation.[342] Hashimoto et al.[342] reacted the NHS ester of biotin with IgG or $F(ab')_2$ in 0.1 *M* sodium bicarbonate to form the Ig-biotin derivative. Linkage of the agent to avidin was achieved by disulfide interchange since the technique was being applied to a toxin A chain. Avidin was reacted at pH 7.6 with a fivefold molar excess of SPDP to obtain the PDT derivative (PDT-avidin). Toxin A chain bearing a free -SH group was prepared by reduction with a low molecular weight sulfhydryl compound and allowed to react with PDT-avidin to produce "A-chain-S-S-avidin". The product was purified by chromatography on Sephadex® G-150 to yield mainly the 1:1 binary conjugate. Incubation of the two binary conjugates in buffer formed the noncovalent ternary conjugate. In an alternative approach, Hashimoto et al.[342] exposed target cells to biotinylated Ig followed by the avidin-bearing agent.

Another approach to noncovalent binding is to utilize the antibody-antigen interaction where high affinities can be observed. The agent has been used as antigen so that antigen-antibody complexes can be targeted to Fc receptor-bearing cells.[343] Alternatively, hybrid antibodies have been synthesized that combine target-antigen-binding

and cytotoxic-agent-binding moieties by oxidative combination of the appropriate F(ab) -SH fragments.[344] However, endocytic uptake by cells may be less than expected for a bivalent antibody if the hybrid is univalent with respect to binding to a cell surface target antigen. On the other hand, the hybrid may serve to deliver agent to a cell surface-binding site for that agent if one is present.

2. Covalent Linkage

a. Direct Linkage between Agent and Antibody

The simplest approach to direct linkage entails a single incubation of agent and Ig along with an activating reagent. The most commonly used such single-stage method has been the formation of amide bonds by use of a water-soluble carbodiimide. This method can be applied to a carboxyl group-containing drugs, such as chlorambucil or MTX, or to a protein agent. Its chief advantage lies in the mild conditions that can be used to achieve coupling, while its chief disadvantage arises from the simultaneous presence of carboxyl and amino groups in proteins. This has the potential for producing cross-linking that may be difficult to control. (The presence of reactive carboxyl and amino groups in the agent can be similarly disadvantageous.) Attempts have been made to control cross-linking by first allowing the carbodiimide to react with the carboxy-containing compound and then introducing the Ig. In preparing daunorubicin-IgG conjugates, Gallego et al.[334] were able to restrict intermolecular cross-linking of IgG to less than 5% (estimated by SDS-PAGE) by using a molar ratio of 1:25:50 for IgG, drug, and ECDI, respectively.

Other conjugation methods have been developed that avoid cross-linkage and homopolymerization. Some involve preparing monofunctional derivatives of the agent or the Ig that are sufficiently reactive to become coupled under the mild aqueous conditions required to prevent denaturation of protein. The strategy is illustrated by the synthesis of active ester derivatives of carboxyl group-containing compounds that do not also possess an unprotected reactive nucleophilic group. The free carboxyl group can be derivatized by reaction with NHS and a carbodiimide. In the second stage, the active ester will react under mild aqueous conditions with nucleophilic groups in the Ig, chiefly amino groups, to produce amide linkages. This method has been applied to MTX conjugation.[345]

Another way of activating a carboxyl group-containing drug is to produce a hydrazide by reaction of hydrazine with either an ester or active ester derivative of the drug. Hydrazide derivatives will react with aldehyde groups to form hydrazones. This has been done with a fraction of the aldehyde groups in the intermediary, polyaldehyde dextran.[346,347] The drug-carrying polyaldehyde dextran was then allowed to react via remaining aldehyde groups with amino groups in an Ig. However, this latter step reintroduces the risk of cross-linking due to more than one Ig molecule reacting with the multiple aldehyde groups present on a given dextran molecule. Alternatively, a hydrazide derivative may be converted to the corresponding azide by reaction with nitrous acid. The azide will react with amino groups in proteins or in polylysine as intermediary. Various homo- and heterobifunctional reagents have also been used for synthesis of antibody conjugates, especially in the linking of other proteins to Ig.[14,18]

b. Linkage through Spacer Arms

Steric hindrance must be prevented if a conjugated agent is to interact with its molecular target while the agent is still bound to Ig. The same approach can be applied to linkage of agents to antibodies, as is used in affinity chromatography to overcome steric hindrance. In situations where the agent is released prior to exerting its cytotoxic effect, a moiety equivalent to a spacer may still be introduced so as to allow linkage bonds of a particular kind, e.g., pH-sensitive.[337,348]

Examples of lysosomotropic spacers are furnished by a series of glycopeptide derivatives of daunorubicin. Monsigny et al.[338] synthesized 2-(1-thio-beta-D-glucopyranosyl)-ethanoyl-L-arginyl-L-leucine (Glc-S-Et-Arg-Leu), the choice of arginine being based on specificity of lysosomal proteases and of leucine on the fact that leucyl daunorubicin is fully active. This intermediate (bearing a free carboxyl group on the leucine moiety) was reacted with NHS and ECDI to form the corresponding active ester which, in turn, was reacted with daunorubicin via the drug amino group to produce a binary amide-coupled drug-spacer conjugate. The sugar ring was then oxidized with periodate to yield a dialdehyde which is capable of coupling to proteins (in this case wheat germ agglutinin) through Schiff base formation. Final stabilization was achieved by reaction with borohydride. Incorporation ratios were low since above 1.5 mol of drug per mole of protein the conjugate became insoluble. The ternary conjugate bound readily to lectin receptors at 4 and 37°C and was more effective than the free drug in inhibiting growth of L1210 cells. A specific competitive effect on both binding and cytotoxicity was observed with di-*N*-acetylchitobiose. Fluorescence microscopy showed membrane labeling after 30 min and fluorescence in clusters after 1 hr. Lysosomal protease susceptibility was shown by preparing solvent extracts of exposed cells and subjecting them to TLC. Derivatives with Rf values similar to free daunorubicin, to leucyldaunorubicin, and to the binary drug-spacer conjugate were observed. The binary conjugate, while taken up by cells slowly, was not cytotoxic and did not release free daunorubicin or a leucyl derivative.

This group later reported on a series of L-Ala-L-Leu peptides spacers with up to four residues, intended to be lysosomotropic.[339] Leu-, Ala-Leu-, Leu-Ala-Leu-, and Ala-Leu-Ala-Leu-daunorubicin were first synthesized. ECDI was used to conjugate succinylated BSA (sBSA) with the amino group of daunorubicin or its peptide derivative. Incorporation was 10 to 21 mol of daunorubicin per mole of sBSA. Succinylation of the protein to block reactive amino groups decreased the extent of polymer formation and about 70% of the conjugate could be isolated in the monomer fraction from Sephadex® 6B chromatography. Incubation of these conjugates with a rat liver lysosomal fraction at pH 5.5 showed that a spacer length (n) of three or four amino acid residues was required for release of drug as determined by HPLC. The linkage was stable in serum, a finding consistent with maintaining the integrity of conjugates in transit to target sites. An i.p. injection of 2 mg/kg of free drug on days 1 and 2 into L1210-cell-inoculated mice showed a 39% increase in life span. Injections of conjugates showed no prolongation when n = 0 or 1, an effect less than the free drug when n = 2, but prolongation of survival of the order of 200% when n = 3 or 4. Also, lower weight loss in the conjugate-treated group indicated a reduction in toxicity compared to the free drug.

A logical refinement of this conjugation procedure was to prepare the active ester of Ala-Leu-Ala-Leu-daunorubicin (and the corresponding adriamycin derivative) and react that with the protein instead of using carbodiimide coupling.[349] Reaction of the free amino group of the peptide with succinic anhydride was used to first introduce a carboxyl group; conversion to the active ester was then carried out with NHS. Conjugation was effected by reaction with protein at pH 9. Conjugates with serum albumin incorporated 10 to 12 mol of ligand per mole of albumin while conjugates with MAB IgG2B and IgG3 incorporated only 3 mol of drug per mole of IgG. The active ester concentration could not be increased because of precipitation. Susceptibility to denaturation may depend on the particular active ester as well as the protein since Kulkarni et al.[345] were able to achieve incorporation ratios of 10 mol/mol of IgG and 90% recoveries of protein using an active ester of MTX.

Shen and Ryser[333] made use of an interesting system for investigating potentially lysosomotropic conjugates. They prepared *N-cis*-aconityl and *N*-maleyl derivatives of

daunorubicin by reacting the drug with the respective acid anhydrides. When the *N*-*cis*-aconityl derivative was conjugated to an amino group-containing solid matrix, the half-life for hydrolysis was 3 hr at pH 4 and more than 96 hr at pH 6 or above. This conjugate had no effect on growth of WEHI cells at pH 7. When *N*-*cis*-aconityl daunorubicin was conjugated to poly-D-lysine using ECDI, the resulting binary conjugate could be taken up by WEHI cells and strongly inhibited growth. (The poly-D-lysine polymer backbone is stable intracellularly, unlike poly-L-lysine.) A binary conjugate of *N*-maleyl daunorubicin and poly-D-lysine could also be taken up by cultured WEHI cells but had no effect on growth, as expected since the maleyl group lacks the *cis*-carboxyl which is responsible for ease of hydrolysis. Analogous binary conjugates could be synthesized with MTX by coupling to poly-D-lysine with and without the digestible spacer, triglycine.[350]

c. Linkage through Multivalent Intermediaries

Limitations on the molar incorporation ratio achievable by direct linkage can be overcome by linkage via multivalent intermediaries such as dextran, polylysine, pGA, and serum albumin.[351] At equimolar incorporations, loss of activity is likely to be less when ligands are bound via an intermediary. However, this has not been systematically investigated. Even though the intermediary may incorporate a large number of substituent molecules, conjugation of a large drug load may alter the properties of the complex such that denaturation takes place.

i. Poly Amino Acids

Early attempts to form conjugates with pGA as the multivalent intermediary used ECDI both to link the drug to the pGA and to link the binary drug-pGA conjugate to Ig. *p*-Phenylenediamine mustard was linked in this way.[352] Later, Hara's group[353] took advantage of the fact that in the unique terminal carboxyl groups a pGA molecule can provide a single site of attachment to Ig. This avoids polymerization arising from attachment of multiple Ig molecules to the intermediary. One approach was first to use SPDP[354] to prepare monopyridyldithio-pGA (R-S-S-pGA) and treat it with DTT to reduce the R-S-S-pGA to the corresponding free -SH compound, HS-pGA. The mixture of pGA and HS-pGA was next treated with thiopropyl Sepharose 6B to bind the HS-pGA by disulfide formation and so allow removal of the unreacted pGA. The washed resin was treated with excess MET to release the HS-pGA by disulfide interchange which, finally, could be reconverted to R-S-S-pGA by reaction with an excess of 2-pyridyl disulfide. An alternative method for introducing the terminal sulfhydryl residue into pGA entailed polymerizing gamma-benzyl-*N*-carboxy-L-glutamate anhydride with cystamine to form a mixture of pGA-CO-CH_2-CH_2-S-S-CH_2-CH_2-CO-pGA and pGA.[355] Reduction with DTT then produced the free -SH compound which was reacted with thiopropyl Sepharose 6B as described above to remove pGA lacking an -SH group. Finally, reaction was carried out with an excess of 2-pyridyl disulfide to protect the thiol and allow for spectrophotometric assay. This formed R-S-S-CH_2-CH_2-pGA of an average molecular mass of 12 kdaltons.

Ternary conjugate formation using R-S-S-pGA to which an agent had been coupled (see below) was carried out as follows.[353] The Ig was substituted with maleimide groups by reacting it with a 20-fold molar excess of SMBE or of the corresponding butyrate, SMBU, which produced a more stable derivative. The average number of maleimide groups incorporated per molecule of Ig was 6.7 (SMBE) and 12.1 (SMBU). Finally, the drug-substituted R-S-S-pGA was reduced with DTT and reacted with the maleimide-substituted Ig to form the ternary conjugate. For daunorubicin conjugation, purification of the ternary conjugate was carried out by starch-block electrophoresis, which will remove contamination by drug-substituted R-S-S-pGA that has not reacted with

Ig. Incorporations of one to three drug-carrying intermediary molecules per molecule of Ig were obtained, depending on the molar ratio (4 to 10) of drug-substituted R-S-S-pGA to maleimide-substituted Ig in the reaction mixture and on the maleimide content of the Ig. Essentially, the same method was used for coupling of drug-substituted R-S-S-CH_2-CH_2-pGA to Ig.[355] The biological behavior of ternary daunorubicin-pGA-antibody conjugates is described under Section V, Current Status.

The method of linkage of a particular agent to pGA-bearing -SH groups in preparation for ternary conjugate formation will depend on the functional groups present in the agent. These workers coupled daunorubicin simply by using a water-soluble carbodiimide since R-S-S-pGA lacks an appropriate nucleophilic group and would not be expected to undergo polymerization. To allow ARA-C to be similarly linked using ECDI, an aminoalkylphosphoryl group was introduced into the drug at the 5′ hydroxyl of the sugar.[356] Linkage to pGA by ECDI was regiospecific and 20 to 30% of the gamma carboxyl groups were substituted. ARA-C was also linked at the 4-amino of the cytosine moiety by reaction with a mixed anhydride derivative of pGA to form an amide bond. In this case, 15 to 20% of the residues were substituted. With both types of conjugates, the remaining free carboxyl groups of pGA could be blocked with 2-aminoethanol and ECDI to convert them to the corresponding hydroxyethyl amides. The 4-amino-group-based linkage was slowly hydrolyzed in aqueous buffer at 37°C; 17% of the drug was released at pH 7.4 after 4 days and 36% at pH 5 after 4.4 days. The alkylamino-group-based linkage was stable in aqueous buffer but was 40% hydrolyzed by phosphodiesterase 1 after 24 hr at pH 7.4. In vitro cytotoxicity tests based on continuous exposure of L1210 cells for 70 hr gave the following order of effectiveness: free ARA-C >> 4-amino-based conjugate > aminoalkyl-based conjugate. Blockage of carboxyls in the aminoalkyl-based, but not in the 4-amino-based, conjugates reduced cytotoxicity. The authors explained these observations on the basis of hydrolytic release of free drug from the 4-amino-based conjugate.

Tumor inhibition was assayed after both single and repeated i.p. doses into tumor-inoculated animals. In contrast to the in vitro results, the conjugates were equal to or more effective in vivo than the free drug. The 4-amino-based conjugate in which the carboxyl charge was neutralized was the most effective, producing some long-term survival with the repeated dose schedule. The mode of in vivo action of these binary conjugates may involve both extra- and intracellular release of drug. Factors determining effectiveness would include "the type and stability of the chemical linkage binding drug to macromolecule, the length of spacer arm, and the nature of the macromolecule itself".[356] It will be interesting to study the properties of ternary conjugates with antitumor antibodies.

ii. Proteins

Use of a protein as a multivalent spacer may be considered in the same category as coupling of a protein agent to Ig, as is dealt with in a following section. The attractiveness lies in the presence in the spacer protein of multiple potential linkage groups, e.g., amino, carboxyl, etc. Serum albumin, the main protein used for this application, possesses desirable characteristics of stability in aqueous media over a fairly wide pH range and of tolerance toward organic solvents to some degree.[351] Incorporation of an agent into a spacer protein and the subsequent coupling of the binary conjugate to Ig may pose problems of cross-linkage and polymerization.

iii. Polysaccharides

Dextran has been used to link several agents to Ig. These include: daunorubicin,[357] bleomycin,[358] mitomycin-C,[359] MTX,[360] ARA-C,[361] FUR,[361] and adriamycin.[13] With most amino group-containing agents, the approach has been to first oxidize the dextran

with periodate to form polyaldehyde dextran. The polyaldehyde dextran is then incubated in aqueous solution with the drug for several hours, followed by the Ig for a further several hours or vice versa. This approach produces a composite Schiff base but leads to polymerization due to the presence of multiple amino groups in Ig. The Schiff bases can be finally treated with borohydride or cyanoborohydride to stabilize the linkage.

An alternative approach has been to utilize dextran hydrazide for coupling of an aldehyde derivative of the agent to Ig.[361] A procedure for preparing dextran hydrazide has been published by Hurwitz et al.,[347] and aldehyde groups can be introduced into agents that possess vicinal hydroxyl groups, e.g., nucleosides, by periodate oxidation. Coupling with dextran hydrazide has been effected by forming a hydrazone with the periodate-oxidized agent followed by linking of the binary hydrazone conjugate to Ig using 0.08% glutaraldehyde.[361] Although the actual reaction may be complex, one terminal aldehyde group of glutaraldehyde would be expected to form a hydrazone with the hydrazide groups of the dextran carrying the drug moiety. The other would form Michael adducts with amino groups in the protein as in typical glutaraldehyde cross-linking. In this synthesis, stabilization by reduction was not carried out.

Carbohydrate moieties in Ig constitute an intrinsic multivalent intermediary since periodate oxidation of the vicinal hydroxyl groups produces multiple aldehydes which can react with amino or hydrazide groups in an agent. However, when aldehyde groups are formed in the carbohydrate moieties of Ig, there occurs the possibility of forming intra- and intermolecular cross-links depending on steric factors. Hemiacetals may form with hydroxyl groups; aldol condensation may occur with another aldehyde group; imines may form by reaction with amino groups in the same or another Ig molecule. In addition to the cross-linking, these reactions can interfere with conjugation of an amino or hydrazide derivative of the agent. However, some of the aldehyde side reactions will be reversible and thus overcome by an excess of agent in the coupling stage. Certain of these considerations also apply to polysaccharide intermediaries.

d. Linkage of Protein Agents to Immunoglobulins

Methods for protein-protein conjugation have been recently and extensively reviewed.[14,18,340,344,362-366] The article by Blair and Ghose[18] lists most types of reactions along with relevant equations. Detailed directions for preparing toxin-antibody conjugates along with important information on safety precautions have been given by Cumber et al.[367] and Domingo and Trowbridge.[368] Here, we will comment briefly on current methodology.

An advance over simple homobifunctional reagents has been to employ heterobifunctional reagents in which two different functional groups possess differential reactivities that can be exploited in conjugation. This provides the ability to substitute a reagent molecule into protein 1 (e.g., the antibody) with the other functional group remaining intact. Ideally, there should be a high yield of the monomeric derivatized protein 1 that can then be coupled with protein 2 (e.g., the toxin). One may also consider this approach in terms of chemical modification of one or both proteins so as to introduce suitable reactive groups. The second stage in conjugation is then carried out under another set of conditions that favor reaction of the newly introduced functional group(s). Depending on the method, the product distribution can be influenced by the concentration ratio of the modified protein 1 and protein 2 if the latter contains more than one reactive linkage site. Keeping the second protein in excess will favor formation of 1:1 conjugates rather than formation of conjugates consisting of two antibody molecules, one toxin molecule, and two or more antibody molecules, etc. Conditions can be chosen to minimize these unwanted products and they can be excluded more definitively by ensuring that protein 2 also has only one site or group that will react

with the substituted protein 1, as is the case with a reduced toxin A-chain-SH or reduced Fab-SH.

An example of a heterobifunctional reagent is the NHS ester of chlorambucil.[364] The *N*-hydroxysuccinimidyl moiety will react with amino groups of protein 1 at low temperature, and then after purification of the derivatized protein, the introduced alkylating moiety will react with amino groups of protein 2 at higher temperatures. If six to eight chlorambucil molecules are introduced into one Ig molecule, then reaction with a four- to fivefold molar excess of a second protein will yield 5 to 10% of a 1:1 conjugate.[367] Another example of the heterobifunctional reagent approach is the use of the NHS ester of *N* (4-carboxycyclohexylmethyl)-maleimide to introduce maleimide groups into a protein which can then react with thiol groups of a second protein such as reduced Ig or F(ab)-SH.[369] As described above, SMBE or SMBU can be used to incorporate maleimide groups into Ig which can subsequently react with a second molecule carrying a thiol substituent to form a ternary conjugate.[353]

SPDP has become widely used to introduce a disulfide group directly into proteins[354] and Ghose et al. have outlined its use in production of antibody conjugates.[14,18] The group introduced is PDT and its extent of substitution may readily be determined by spectophotometry. Depending on the protein and reaction conditions, a sixfold molar excess of SPDP can result in the substitution of about three groups per protein molecule.[354,368] The PDT group is susceptible to a disulfide interchange reaction with another protein possessing a free -SH group. The final product will be a conjugate with a disulfide bridge linking the two proteins. The second protein with a free -SH group can be produced in several ways, notably by using SPDP to introduce PDT groups as above, and then reducing them. With PDT groups, the interchange reaction favors conjugate formation.

In conjugating whole toxins, the free -SH groups would normally be incorporated into the Ig rather than into the toxin to avoid reductive cleavage into toxin A and B chains or polymerization.[367] Introduction of free -SH groups into Ig can be achieved by reducing PDT-Ig under mild conditions with DTT without splitting interchain disulfide bridges.[334] With an average of two reactive groups per protein (-SH in one and pyridyldithio in the other) and equimolar reaction mixtures, a 20 to 40% yield of a 1:1 conjugate can be obtained by disulfide interchange coupling.[367] In conjugating a toxin A chain, the procedure would be to react PDT-Ig with the reduced single chain toxin possessing a single -SH group. With two PDT groups per Ig and a two- to threefold molar excess of A-chain-SH, the yield of a 1:1 conjugate is of the order of 50%.[367] The reaction of an S-sulfonated protein with Fab-SH is another example of coupling via an -SH group of which there is only one per molecule.[370]

N-succinimidyl iodoacetate can be reacted in neutral buffer with epsilon amino groups to introduce iodoacetyl groups into a protein.[351,367,371] Tritiated reagent may be used to monitor incorporation which can be limited to a few sites, e.g., two in the case of ricin[372] and the MAB studied by Garnett et al.[351] Analogous reagents include the anhydride and *N*-hydroxysuccinimide ester of bromo-acetyl-*p*-aminobenzoic acid.[364] Coupling of the haloacetyl derivative will occur smoothly at pH 7 under nitrogen with a second protein molecule carrying a free -SH group, e.g., produced by reduction of a PDT-substituted protein.[351] This produces a thioether bond rather than a disulfide. The yield of 1:1 conjugate is also between 20 and 40%. Whole toxin conjugates prepared with iodoacetylated toxin will retain the original disulfide linkage between the A and B chains, whereas the reaction between iodoacetylated Ig and a toxin A chain produces a thioether-linkage.

C. Purification of Conjugates

The following comments will illustrate some of the difficulties in obtaining homo-

geneous purified preparations and, more broadly, underline the desirability of designing linkage methods that facilitate production of well-defined conjugates. If a polyclonal Ig is used, it will be heterogeneous and this mixture of molecular forms will result in a corresponding mixture of conjugate species. Whatever amino acid side chain or carbohydrate residue is chosen for the linkage reaction, there will be variations in the number and distribution in different Ig molecules. This will lead to variations in the extent of agent incorporation which will be superimposed on variations arising from the linkage reaction itself as discussed below. The use of MAB avoids heterogeneity in conjugate structure arising from heterogeneity in Ig structure. However, heterogeneity can still arise with monoclonal Ig. Linkage sites, e.g., a particular amino acid side chain, will occur in different locations over the accessible surface of the Ig, and differing neighboring groups will influence the chemical reactivity at each individual site. A given site in a given molecule may or may not become substituted, resulting in a distribution in overall incorporation for a population of Ig molecules. Also, the chemical linkage reaction may not be specific and incorporation may occur at more than one class of site, with possible variation in retention of inherent agent activity. A further ramification of this heterogeneity is the fact that some incorporation may occur in a variable manner in the antigen-binding site so as to affect antigen binding. Thus, the population of conjugate molecules may vary, not only with respect to total molar incorporation per Ig molecule, but also with respect to affinity for antigen.

Differences in charge, size, hydrophobicity, etc. may not be adequate for separation of the mixture of conjugate species by conventional methods. In practice, purification has been carried out in many cases simply by gel filtration to remove unbound agent, chemical reaction side products, and grossly aggregated Ig. This technique may fail to effectively resolve the desired conjugate from polymers of Ig or from unbound high molecular weight agents such as protein toxins. However, isoelectric focusing, preparative HPLC, various gel electrophoresis techniques, etc. can achieve partial if not complete resolution. Affinity techniques based on antigen binding are in principle capable of eliminating conjugate molecules with little or no binding capacity, while those based on agent binding can eliminate Ig molecules with little or no agent incorporated.

In some cases, purification techniques with superior resolution have been used. For example, Uckun et al.[373] used protein A-Sepharose to purify a conjugate of pokeweed antiviral protein and a monoclonal IgG1. Unconjugated toxin was not adsorbed. The IgG1 bound weakly to protein A, allowing elution under mild conditions, i.e., with pH 5.5 citrate. A disadvantage of this approach is that it will not resolve free Ig and conjugate unless there is a distinct difference in the affinity for protein A. In another instance, Kato et al.[355] used preparative disc PAGE to purify drug-pGA-Ig conjugates, leading to reported purities for the ternary antibody conjugates of the order of 90%.

Purification of toxin-antibody conjugates has been outlined by Cumber et al.[367] With protein-protein conjugates, simply eliminating a low molecular weight fraction by gel filtration will not resolve various polymers — trimers, tetramers, etc. — hence the desirability of linkage methods that optimize production of 1:1 conjugates. Gel filtration using Sephadex® G-200 or Sephacryl S-300 gels will allow separation of unreacted toxin chains or whole toxins (which are weakly absorbed by the matrix) and higher polymers. Protein A absorbants will allow removal of free toxins since they retain antibodies if they are of the appropriate class. Conversely, free antibody can be removed by treating conjugates with an adsorbant with affinity for the toxin moiety.

D. Characterization of Conjugates

Characterization of conjugates includes: (1) extent of incorporation of agent in Ig; (2) extent of preservation of the inherent activity of the conjugated agent; and (3)

extent of preservation of antibody activity of the Ig. The objective for therapy is selective toxicity to a target tissue in vivo. Thus, testing of conjugates has tended to focus on measurements of growth inhibition of cells in culture or inoculated into experimental animals, without extensive prior chemical or immunological characterization. Problems in characterization can arise from the heterogeneity of conjugate preparations. However, proper characterization should aid in the design of optimally active conjugates.

1. Cytotoxic Agent Incorporation and Activity

If the agent possesses a chromophoric group, incorporation may be determined spectrophotometrically once the molar absorption coefficient has been determined. If a radioactive agent is available or can be synthesized, isotope counting can provide a sensitive method for measuring incorporation. Toxin-antibody conjugates have been characterized by nonreducing SDS-PAGE that can resolve molecular species consisting of toxin linked to Ig in molar ratios of 0:1, 1:1, 2:1, 3:1, etc.[367,368] Also, toxin incorporation can be determined by radioiodinated toxin[367] or by solid-state RIA using antitoxin antibodies.[374]

Assay of retention of agent activity may be relatively straightforward if the agent can interact with the molecular target when it is in bound form. Assays that may be applicable include enzyme inhibition, complexing with DNA, etc. The activity in conjugate form of an alkylating agent such as chlorambucil has been determined spectrophotometrically[375] and by a fluorescence method.[376] These methods can measure both the extent of incorporation and retention of alkylating activity. Advantage may be taken of direct interaction with a target molecule as in the case of MTX which binds very strongly to DHFR. The capacity of the bound antimetabolite to combine with the enzyme is determined by measuring the inhibition of enzyme activity.[345] If a decrease in activity is observed, such determinations do not distinguish between a general decrease in binding affinity for conjugated drug and a total incapacity to bind for a fraction of the conjugated drug. Binding assays using an immobilized enzyme should help in characterization but have not been applied to MTX conjugates. In the case of disulfide-linked toxin conjugates, the toxin moiety can be assayed by measuring protein synthesis with a reticulocyte lysate.[377,378] When release of a bound agent is required for cytotoxicity, then assay methodology can be designed to take this into account. Measurement of inhibition of growth of bacterial cells in culture that are sensitive to the agent has been used but cannot always be extrapolated to effects on human or experimental tumor cells.[359]

2. Antibody Activity

Numerous techniques such as indirect immunofluorescence with target cells and ELISA have been used to evaluate conjugates for retention of antibody activity and this topic is beyond the scope of the present review. Lindmo et al.[379] have recently described a convenient analysis to establish the immunologically active fraction and the affinity constant. Their procedure is based upon data from the measurement of the binding of isotopically labeled antibody to target cells. This would be useful in distinguishing the presence of a fraction of conjugate molecules that is incapable of binding to target antigen and might be eliminated from the preparation by an affinity technique.

E. Binding of Antibodies to Drug-Carrying Liposomes

The amount of a drug that an antibody can carry may be greatly increased if the antibody is bound to liposomes into which the drug is incorporated instead of being linked directly to the drug. This is because lipid vesicles can contain many more drug

molecules than can be incorporated into an Ig molecule. Drugs do not need any chemical manipulation for liposomal encapsulation and they are protected by the liposomal membrane during transit. Furthermore, the altered pharmacokinetic and metabolic properties of liposome encapsulated agents (e.g., slower clearance and catabolism) may be therapeutically advantageous.[380,381] The stability of the antibody-liposome bond is of prime importance. Several methods for coupling Ig to liposomes via a stable covalent linkage have been reported,[382-387] but the efficiency of binding of some is very low.[383,387,388] In several other methods,[386,387] dialysis in the presence of a detergent is used to bind Ig onto lipid vesicles, but the entrapped substance is likely to leak out. Martin et al.[385] and Goundalkar et al.[389] have described methods which have higher efficiency of binding Ig and which avoid dialysis in the presence of a detergent. Ghose et al. and others[381,390] have demonstrated that, at least in vitro, antibody-linked drug-carrying liposomes aggregate around and bind to target cells. However, whether the liposomal content can reach intracellular target molecules in vitro or in vivo remains to be conclusively demonstrated.[380,381] Recently, Matthay et al.[391] showed that antibody-coated small unilamellar vesicles containing MTX-gamma-aspartate aggregated around and were endocytosed by target mouse tumor cells. The size of liposomes appears to be critical since adequate endocytosis could take place only with small unilamellar vesicles. Results of studies on the tumor uptake of such targeted small unilamellar vesicles in vivo will be of interest.

V. CURRENT STATUS OF CYTOTOXIC-AGENT-ANTIBODY CONJUGATES

In this section, the authors will discuss the properties of selected conjugates of low molecular weight drugs and protein toxins that illustrate the scope of the field, emphasizing more recent reports.

A. Antibody-Bound Drugs

Drugs of greatest current interest for antibody conjugation include daunorubicin and adriamycin, MTX, chlorambucil, vindesine, and mitomycin C. Reports have also appeared on bleomycin, FUR, ARA-C, and neocarzinostatin. Other drugs that mainly were the subject of earlier studies have been reviewed elsewhere.[12,14,340] These include phenylenediamine mustard,[392] Trenimon,[292] and melfalan.[393]

1. Chlorambucil

Chlorambucil can bind noncovalently to Ig and possesses a carboxyl group which allows for application of various covalent linkage methods.[14,340] Both covalent and noncovalent antibody conjugates that retain drug and antibody activity have been studied by several groups.[128,392,394-402] Factors affecting noncovalent binding have been discussed.[403-406] Covalent conjugates using dextran have also been prepared.[392] The carbodiimide-mediated linkage gave recoveries of antibody activity that varied depending on the antibody used for conjugation. With polyclonal IgG against EL4 cells, up to 40 mol/mol were incorporated with substantial retention of anti-EL4 antibody activity.[398] In addition to ECDI-mediated linkage,[398] the isocyanate derivative of chlorambucil has been reported to react with an anti-CEA antibody to yield a conjugate with 25 mol of chlorambucil per mole of Ig and retention of alkylating activity,[376,402] but the antibody activity was not reported. When this conjugate was assayed in vitro on a human colon adenocarcinoma line with continuous exposure, the order of effectiveness was specific conjugate > chlorambucil > a mixture of drug and specific antibody > nonspecific conjugate. The antibody alone was not cytotoxic. Short-term exposure to the agent also showed that the conjugate was more potent than the free drug. The conjugate was

no more effective than the free drug on a non-CEA-producing cell line. In vivo assays were not carried out.

Using the noncovalent method of linkage,[403] between 100 and 500 mol of active chlorambucil per mole of Ig (after dialysis against PBS overnight) were bound to two affinity-purified polyclonal anti-idiotype antibodies against two IgM-secreting human lymphoblastoid cell lines (RPMI-6410 and RPMI-8392).[401] However, the extent of retention of antibody activity in the conjugate was not reported. A complement-independent ^{51}Cr release cytotoxicity assay showed that the specific conjugate was at least threefold more cytotoxic toward target cells than nontarget cells and more effective than free drug, drug plus antibody, or drug plus normal rabbit Ig.

2. MTX

MTX was one of the earliest choices for preparing drug-antibody conjugates.[12,14,340] This antimetabolite is highly potent; its complex formation with the target enzyme DHFR is virtually stoichiometric, although an excess is required intracellularly for effective action.[407] MTX contains both amino and carboxyl groups, but manipulation of the carboxyl groups is less likely to interfere with enzyme inhibition.[345] The drug is commercially available with tritium labeling, either in the glutamate or *p*-aminobenzoyl moieties so that isotope methods can be employed to determine incorporation and uptake and catabolism of its conjugates.[295,307,408] MTX was rendered selectively toxic to tumor cells when covalently coupled by an active ester method to a rabbit IgG antibody against a TAA on the surface of mouse EL4 lymphoma cells; this conjugate inhibited tumor growth more effectively in vivo than did free MTX or MTX linked to normal rabbit globulin (NRG).[345]

More recent studies have dealt with conjugates synthesized with antimelanoma antibodies and their testing in a human solid tumor xenograft model, which is more pertinent to the clinical setting than ascites tumor models. Antimelanoma MAB 225.28S reacts with a high molecular weight MAA on the surface of human melanoma cells. Using radioiodine-labeled MAB 225.28S which had been purified by ion-exchange chromatography and gel filtration, Ghose et al. have shown that intravenously injected antibody selectively localizes in xenografts of human melanoma in nude mice.[139] It was also shown that sera from melanoma-bearing nude mice did not obstruct binding of the specific MAB to melanoma cells. MTX was covalently linked to MAB 225.28S to produce a conjugate with an incorporation ratio of 5 to 6 mol/mol of IgG.[302] It gave a single band on SDS-PAGE, and there was no decrease in membrane immunofluorescence titer of antibody activity at the incorporation ratios used. Conjugated MTX was approximately half as inhibitory toward DHFR as the free drug. In human melanoma M21 xenografted nude mice, multiple i.v. injections of the MTX-MAB conjugate inhibited the tumor more effectively than the MAB, the free drug, or the drug-linked to normal mouse IgG.[302] There was also no tumor inhibition by a mixture of MTX and the MAB. A polyclonal rabbit antihuman melanoma IgG carrying equivalent amounts of MTX was tumor inhibitory but less so than the MTX-MAB conjugate. In vitro experiments showed that the MTX-MAB conjugate was less cytotoxic than free MTX, a result that has been obtained with other conjugated drugs.[292,409]

Garnett et al.[351] prepared a conjugate of MTX and an antibody against a human osteogenic sarcoma cell line, MAB 791T/36, which had been purified by chromatography on protein-A Sepharose. They used HSA as a multivalent intermediary. The methodology entailed introduction of sulfur-containing moieties preparatory to forming a thioether linkage. HSA was reacted with SPDP to yield PDT-HSA, with an incorporation ratio of 2.[354] MTX was incorporated into the PDT-HSA intermediary using ECDI at a molar ratio of 100:70:1 (ECDI:MTX:PDT-HSA). The monomer peak obtained by gel chromatography of PDT-HSA-MTX (molar incorporation

ratio 32 mol of MTX per mole of HSA) was used for synthesizing the ternary conjugate. PDT-HSA-MTX was reduced to the corresponding free -SH derivative by treatment with DTT and reacted in a 4:1 molar ratio with iodoacetyl-IgG (molar incorporation ratio 2) which had been prepared by reaction of IgG with *N*-succinimidyl iodoacetate.[371] Fractogel TSK HW55s partially resolved MTX-HSA, unconjugated IgG, the ternary conjugate, and higher polymers. ^{131}I labeling of HSA aided in characterizing column fractions and allowed calculation of the molar ratio of HSA to IgG in addition to MTX in the final product. The final stoichiometry in the pooled ternary conjugate fractions was reported to be 1 to 3 mol of MTX-HSA (each carrying 32 mol of MTX) per mole of MAB 79IT/36. A competition assay using flow cytofluorimetry showed that 28% of antibody activity was retained after conjugation.

In a cytotoxicity assay based on ^{75}Se-methionine incorporation in vitro, MTX bound to HSA was much less toxic than free MTX toward either target or nontarget cells. MTX in the ternary MAB conjugate was about as toxic as the free drug toward target cells and as toxic as MTX-HSA toward nontarget cells. Antibody alone had little effect on either type of cell. Cytotoxicity assay based on pulse exposure to the test-agent of 15 min followed by culturing in test agent-free medium showed that the ternary conjugate was more toxic than free MTX toward target cells but essentially nontoxic toward nontarget cells. Finally, a clonogenic assay with 5 days of continuous exposure gave I_{50} values of 4 ng/mℓ for both MTX and the ternary conjugate. A competition experiment with free antibody showed the cytotoxicity was strictly dependent on binding of the drug-carrying specific antibody to the cells.

Embleton et al. have evaluated the effectiveness of a MTX-HSA-MAB-791T/36 conjugate on human sarcoma xenografts in nude mice.[410] Multiple doses of test agent of 2.5 mg/kg (MTX equivalent) were given up to day 22 after s.c. tumor inoculation. The tumor diameters at day 32 were 5.6, 3.1, and 8.6 mm when the test agents were MTX, MTX-HSA-MAB, and PBS, respectively. However, six of ten mice treated with free MTX died, whereas there were no toxic deaths in the conjugate-treated group. Garnett and Baldwin have recently improved the synthesis of MTX-HSA-MAB conjugates.[410a] They omitted the SPDP-based introduction of a free -SH group in the HSA and instead reduced the MTX-HSA binary complex itself with DTT to provide the -SH site for conjugation with iodoacetylated antibody. Up to 38 mol of MTX were incorporated per mole of HSA and the ternary conjugate retained 32 to 35% of antibody activity. ^{75}Se-methionine uptake and colony inhibition assays demonstrated that the new conjugate was more toxic than MTX toward antigen-containing cell lines and was more toxic than the conjugate produced by their former method.[351] Separation of different molecular weight fractions by HPLC showed that all were cytotoxic.

Instead of HSA, Manabe et al.[360] have used a dextran-based method to conjugate MTX to IgG which is essentially the same as that used for bleomycin[358] and for mitomycin C.[359] Polyaldehyde dextran was incubated with a murine anti-HLA IgG for 24 hr followed by addition of MTX and further incubation for 24 hr giving the composite imine product. Finally, stabilization was achieved by borohydride treatment. The best MTX incorporation was approximately 9 mol/mol of IgG, not very high considering the use of the multivalent spacer. Inhibition of DHFR activity was used as a gauge of retention of drug activity. The incorporated MTX assayed by spectrophotometry retained 46% of its DHFR inhibitory activity. Membrane immunofluorescence assay showed retention of 85 to 90% of antibody activity.

Cytotoxicity assays used two bases for comparison. In one, more conjugated MTX than free MTX was added to cell suspensions to compensate for the apparent loss of DHFR inhibitory effect on conjugation. Cytotoxicity assay after 3 days of continuous exposure to the test agents revealed that the conjugate was 18-fold more toxic to target

BALL-1 cells than the free drug and 7-fold more toxic than the corresponding nonspecific Ig conjugate. All these test agents had similar toxicity toward nontarget cells. Thus, conjugation increased cytotoxicity irrespective of specificity. Also, the superiority in vitro over free MTX was in contrast to the behavior of the conjugate in which MTX was directly linked to antimelanoma antibodies studied by Ghose et al.[302]

The second set of assays entailed a 2-hr pulse exposure to equimolar concentrations of MTX, free or conjugated. The I_{50} for the specific conjugate measured against HLA-containing NALL-1 cells was 1.24×10^{-6} *M* and measured against HLA-lacking NS-1 cells was 2.9×10^{-6} *M*, the difference being statistically significant. Free MTX was equally cytotoxic toward these cell lines, having an I_{50} around 10^{-6} *M*. Binary MTX-dextran conjugates were not investigated. Thus, the cytotoxic properties can vary substantially depending on the assay conditions. After pulse exposure, the specific conjugate was not a great deal more effective against target cells than against nontarget cells. It was suggested that the HSA intermediary lends itself better than dextran[411] to development of improved lysosomotropic linkages.

The approach to conjugation of MTX taken by Manabe et al.[360] was distinctive in that it involved reaction at the pteridine ring moiety. Kulkarni et al.[345] had conjugated MTX through its carboxyl groups because of the importance of the pteridine ring in interaction with DHFR. In this study by Manabe et al.,[360] it was possible to synthesize cytotoxic conjugates using the amino groups of the pteridine ring. Moreover, the bound drug was still at least partly capable of interacting with DHFR and the authors pointed out that possibly only the MTX molecules linked via the 2-amino rather than the 4-amino group are active, the 4-substituent being the more critical one. Linkage through the latter group could contribute to loss of ability to bind to the active site of DHFR after conjugation. Cytotoxicity could be postulated to occur in the cell culture assay due to release of free drug by cellular catabolism, but this would not explain the DHFR inhibition findings with the intact conjugate.

Shen and Ryser[343] have developed a system for targeting to Fc receptor-bearing cells based on the fact that Fc receptors will bind antigen-antibody complexes. A polyclonal rabbit anti-HSA antiserum was used to form immune complexes with a binary conjugate of tritiated MTX and HSA (18 mol of drug per mole of HSA). Cytotoxicity was assayed by exposing monolayer cultured cells to test agent and then counting cells after 5 to 10 days growth. Tumor cells bearing Fc receptors (lines M5076 and WEHI) became associated with significant amounts of tritium and were killed only in the presence of both MTX-HSA (30 n*M* with respect to MTX) and antiserum, not MTX-HSA alone. That the cell-associated radioactivity represented uptake was shown by the fact that MTX-transport deficient cells as well as cells capable of taking up free MTX were inhibited from growing by MTX-HSA and antiserum. Extracellular release of MTX from the HSA could not have led to inhibition of the transport deficient cells. These studies also showed that phagocytic and nonphagocytic cell lines with Fc receptors were equally susceptible to HSA-conjugated MTX plus antiserum. Protein A, which blocks the Fc interaction with the surface receptor, counteracted cytotoxicity, as did excess unsubstituted HSA.

3. *Anthracycline Glycosides*

Daunorubicin has been conjugated by several methods to different macromolecular carriers.[357] Adriamycin has been less well studied and behaves differently toward certain linkage procedures. An important early comparative study by Hurwitz et al.[412] showed that the homobifunctional reagent glutaraldehyde could form conjugates of daunorubicin by linking presumably via the drug amino group and protein amino groups, but with serious aggregation problems. Single-stage coupling with a water-soluble carbodiimide resulted in substantial loss of activity. Periodate-based coupling

turned out to be the best of the three approaches tried at that time. Direct conjugation was achieved by oxidizing the sugar residue and forming imines with protein amino groups, resulting in incorporation of 2 to 5 mol of drug per mole of Ig. Stabilization was accomplished by treatment with borohydride. The chemical structure of the linkage group resulting from the periodate method has not been well worked out. It has been suggested[347,357] that oxazolidine derivatives are formed or that not all imines are reduced since daunorubicin conjugated by a nonhydrolyzable single sulfur link did not exhibit drug activity in vitro.[347] A number of other conjugation approaches applied to this drug have also been outlined in the section on methods of linkage.[333,338,339,349,353]

Several glutaraldehyde-based direct conjugation studies illustrate different approaches to targeting. Sehon and co-workers coupled daunorubicin to a goat antifibrin antibody, the rationale being that fibrin is present in both animal and human tumors and thus may be a suitable target.[413] The conjugate had 1 to 2 mol of drug per mole of IgG; antibody activity was retained. They used a ^{131}I-labeled antibody to demonstrate that localization occurred in vivo.[414] In vitro cytotoxicity assay on a methylcholanthrene-induced guinea pig sarcoma revealed the conjugate to be equally effective as free daunorubicin.[413] Multiple intratumoral injections into established sarcomas in guinea pigs led to complete tumor rejection in 50% of the animals. Neither free drug nor antibody inhibited tumor growth. While tumor inhibition after local injection could be due to sustained release of free drug at the tumor site or release intracellularly after endocytosis, these authors pointed out that tumor immunity induced by conjugate action on tumor cells could also be involved.[413]

To minimize polymerization and precipitation of Ig during aldehyde-based conjugation procedures, Belles-Isles and Page[415,416] subjected daunorubicin and polyclonal or monoclonal rabbit antihuman CEA IgG to a short incubation at 37°C with 0.01% glutaraldehyde at pH 7.2. They obtained a conjugate with an incorporation ratio of 2 mol/mol of Ig. Antibody activity was not reported. Three different in vitro assays, including continuous and pulse exposures, revealed the conjugate to be the most potent inhibitor. For in vivo assay, this group injected intraperitoneally every other day for 30 days a polyclonal anti-CEA-IgG-daunorubicin conjugate (5 mol of drug per mole of Ig) into nude mice carrying xenografts of LoVo cells measuring approximately 4 mm in diameter.[417] The conjugate, free antibody, and free drug were not significantly different from PBS controls during the first 30 days. However, growth of tumors in conjugate-treated animals then leveled off during next 40 to 50 days, before finally increasing again. Prior studies had demonstrated the localization of labeled anti-CEA antibody in human colonic carcinoma xenografts in nude mice.[418-420]

A comparative study of four different direct linkage groups has been carried out by Gallego et al.,[334] using the same mouse antihuman osteogenic sarcoma IgG2b 791R/36 used by Embleton et al.[421] to prepare MTX conjugates. Conjugates 1 and 2 were prepared from 14-bromodaunorubicin. For conjugate 1, the coupling procedure was based on that of Zunino et al.,[335] i.e., reaction of a 25-fold molar excess of 14-bromodaunorubicin with amino groups of the Ig at pH 7.5. Zunino et al.[335] had shown that protein conjugates prepared with this derivative inhibited colony formation by HeLa cells, but to a substantially lower extent than the free drug. For synthesis of conjugate 2, SPDP was first used to introduce three to four -SH groups into the IgG; reaction at pH 4.5 with a tenfold molar excess of bromodaunorubicin produced a thioether-linked conjugate. The bromo derivative was reported not to react with protein amino groups under these conditions.[334] Another linkage method entailed incorporation of an acid labile *cis*-aconityl spacer linked to both daunorubicin and Ig by amide bonds to form conjugate 3. Coupling at pH 7 was carried out by adding ECDI to IgG mixed with a 25-fold molar excess of carboxy drug. Conjugate 4 was similarly prepared, except that the spacer was a succinyl group which should not be as readily

susceptible to hydrolysis in the lysosomal milieu.[333] The incorporation was 3 to 4 mol of drug per mole of IgG for all daunorubicins. Three assays were used to measure retention of antibody activity: direct and indirect membrane immunofluorescence and competitive inhibition of binding of fluorescin isothiocyanate (FITC)-labeled antibody by conjugate. Conjugate 1 showed complete retention of antibody activity in all assay systems. Conjugates 2 and 3 showed some loss of antibody activity, the extent varying with the assay method. In vitro cytotoxicity assay based on ^{75}Se-methionine incorporation, either with 24-hr continuous exposure or after pulse exposure for 30 min, showed that the succinyl-linked conjugate had no cytotoxicity. After continuous exposure, the other three conjugates were approximately tenfold less potent than the free drug and could not discriminate between target and nontarget cells. Some target cell selectivity could be seen after 30 min pulse exposures to all three conjugates. Based on retention of antibody activity, cytotoxicity, and selectivity, the *cis*-aconityl conjugate was thought to be the most effective.

The intermediary, pGA, has been used to link daunorubicin to an affinity-purified polyclonal anti-AFP antibody.[353,355] The conjugation method was as outlined under Section IV, Methods of Linkage. ECDI was used for coupling of the drug to PDT-pGA (average molecular mass 13 to 15 kdaltons) using a molar ratio of ECDI to daunorubicin to R-S-S-pGA equal to 85:13:1. In the subsequent reaction to form the ternary daunorubicin-pGA-Ig conjugate, daunorubicin-substituted R-S-S-pGA was reacted with maleimide-substituted anti-AFP Ig or with an equivalent amount of maleimide-substituted normal horse Ig (nIg) for the control conjugate. The incorporation was 10 to 20 mol of drug per mole of Ig, depending on the molar ratio (4 to 10) of daunorubicin-substituted R-S-S-pGA-to-maleimide-substituted Ig in the reaction mixture. The extent of retention of antibody activity was not reported. A cytotoxicity assay in vitro after continuous exposure of AH66 hepatoma cells to agents for 18 hr showed that the order of effectiveness was nIg < anti-AFP < daunorubicin, daunorubicin-pGA, daunorubicin-pGA-nIg < daunorubicin-pGA-anti-AFP. Normal Ig at a concentration equal to that used for the highest level of conjugate had no effect, whereas 30 μg/mℓ of drug bound in the specific ternary conjugate gave virtually total inhibition of growth. In an in vitro-in vivo assay, AH66 ascites tumor cells were exposed to the test agents in vitro at 37°C for 30 min and then inoculated intraperitoneally into syngeneic rats. The longest survival of tumor-inoculated rats was observed when the cells had been exposed to the specific ternary conjugate, namely, 53 days compared to 18 days for recipients of PBS-control cells. The survival of rats that received AFP- or daunorubicin-treated cells was also prolonged to 35 and 45 days, respectively. There was no synergism with a mixture of anti-AFP Ig and free daunorubicin. The nonspecific binary and ternary conjugates were about as effective tumor inhibitors as the free drug. When tumor-inoculated animals were given multiple doses of test agents over a 9- or 15-day period, the specific ternary conjugate gave the greatest prolongation of survival, including a proportion of long-term survivors.

The cytotoxicity observed with this ternary IgG conjugate, in which daunorubicin was linked to pGA by using ECDI, is in contrast to the observed loss of drug activity after ECDI-mediated direct linkage to an antibody.[412] It is possible that the cytotoxicity of the ternary conjugate is due to susceptibility to hydrolysis of the amide bond involving the gamma carboxyl of glutamate, releasing free drug, or that pGA can be hydrolyzed intracellularly to produce a cytotoxic fragment comprising a gamma-glutamyl derivative of the drug. It is also pertinent to determine whether the intact ternary conjugate can interact with DNA in vitro. Tsukada et al.[353] pointed out that the approach taken by Shen and Ryser[333,422,423] in comparing cytotoxicity and uptake of poly-L- and poly-D-lysine conjugates could be applicable to elucidating the mode of action of this pGA-based conjugate.

Daunorubicin was also coupled to a 12-kdalton pGA derivative synthesized by polymerization with cystamine.[355] ECDI incorporated 6.5 mol of drug per mole of R-S-S-CH_2-CH_2-pGA. Ternary drug-pGA-antibody conjugates were again prepared with maleimide-substituted anti-AFP and with nIg for the control conjugate. Preparative disc PAGE was used to achieve a reported purification of approximately 90%. The incorporation ratio of drug to Ig was 7 to 7.5 and almost all antibody activity was retained as determined by a precipitation assay with ^{125}I-labeled AFP. In vitro cytotoxicity assay after continuous exposure of AH66 cells for 48 hr to the test agents showed the order of effectiveness to be specific ternary daunorubicin-pGA-anti-AFP > daunorubicin = daunorubicin-pGA = daunorubicin plus anti-AFP IgG = daunorubicin-pGA plus anti-AFP IgG > anti-AFP = daunorubicin-pGA-nIg. There was no effect with nIg. In contrast, with a non-anti-AFP-secreting cell line, AH272, the specific and nonspecific ternary conjugates were equally cytotoxic. However, this effect was slightly less than that of the free drug under these conditions. Neither nIg nor anti-AFP Ig had any effect on this cell line.

Dextran has also been used as an intermediary to increase daunorubicin incorporation. The methodology was an extension of that used by Bernstein et al.[424] to prepare binary daunorubicin-dextran conjugates. Polyaldehyde-dextran of a molecular mass of 10 kdaltons was incubated with daunorubicin for 20 hr and then affinity-purified anti-Yac IgG was added for a further 20 hr, followed by borohydride.[425] The drug incorporation was 2 to 3 mol/mol of dextran and 25 mol/mol of IgG. There was a 40% loss of antibody activity in the conjugate. An early ternary conjugate was less effective than the free drug in vitro and was no better than the binary dextran or ternary nonspecific Ig conjugate in vivo at high doses. Subsequently produced ternary conjugates utilizing both whole Ig and $F(ab')_2$ from an affinity-purified polyclonal horse anti-rat AFP IgG[411] had a substantial loss of antibody activity, but in an in vitro assay involving continuous exposure of AH66 cells to test agents for 48 hr, the specific conjugate was claimed to be 100 times more cytotoxic than a mixture of antibody and drug unlinked, which showed modest synergism. The nonspecific conjugate was no more effective than the free drug. Preincubation of cells with the specific conjugate prior to inoculation into rats led to five out of ten long-term tumor-resistant survivors, whereas all control animals died. The i.p. administration of the specific conjugate to tumor-bearing rats gave an average survival of 64 days compared to 16 days for control animals. Average survival times ranged from 33 to 45 days for rats given free drug, specific antibody, or a mixture of the two. It was pointed out by the authors that low levels of AFP in the group receiving the conjugate showed that AFP-producing tumor cells were being selectively inhibited.

Using dextran, Hurwitz et al.[426] could incorporate 50 mol of daunorubicin per mole of an affinity-purified anti-idiotypic antibody or its $F(ab')_2$ fragment directed against a clonally expressed cell-surface IgM of a B-cell lymphoma. The ternary antibody conjugate exhibited an approximately 50% loss of binding to target cells. In vitro cytotoxicity assay based on inhibition of tritiated thymidine uptake by 38C cells showed the order of effectiveness to be free drug > specific $F(ab')_2$ conjugate > specific whole Ig conjugate = nonspecific Ig conjugate. In i.p. tumor-bearing animals, the specific conjugate was more effective than the free drug when given intraperitoneally but not much better than the free drug when given intravenously. There was a reduction in systemic toxicity of conjugated daunorubicin so that higher doses could be given, producing effective tumor inhibition. At these higher dose levels, the specific conjugate was more effective than the nonspecific conjugate, producing cures in a proportion of animals. $F(ab')_2$ conjugates were less effective than whole Ig conjugates, and increasing the tumor burden above 10,000 cells also lowered the effectiveness of conjugates.

Pimm et al.[427] conjugated 18 to 28 mol of adriamycin to a monoclonal IgG2b against

rat mammary carcinoma Sp4 following the method described for linking daunorubicin to anti-Yac IgG. An in vitro-in vivo assay, in which test agent-pretreated Sp4 cells were inoculated into rats, showed that tumor growth was retarded by the specific conjugate but not by free adriamycin or a nonspecific conjugate. When s.c. tumor-inoculated rats were treated with multiple i.p. doses of test agent, dosages of the order of 500 $\mu g/kg$ for the free drug were required to inhibit tumor growth significantly, but the specific conjugate exerted a distinct effect at adriamycin doses of 30 to 75 $\mu g/kg$. Survival ranged from 30 to 50 days for control animals and from 60 to 90 days for animals treated with the specific conjugate. Antibody alone had no effect on tumor growth and synergism between free drug and antibody was not observed. These authors concluded that this conjugate and test system gave results superior to those obtained by Hurwitz et al. with anti-Yac antibodies.[425] There was evidence for preferential tumor localization of radiolabeled antibody.

Ghose et al.[13,264] successively reacted polyaldehyde dextran T40 with adriamycin and antimouse renal cell carcinoma IgG without borohydride reduction. There was no loss of drug from the conjugate on repeated dialysis. Adriamycin-dextran-IgG conjugates had less systemic toxicity than the free drug. When renal cell-carcinoma-bearing mice were given i.v. injections of equitoxic doses of test agents for 7 successive days, the order of antitumor effect was specific conjugate > nonspecific conjugate > free drug. Only the specific conjugate could produce long-term survival in a proportion of treated mice.

4. Vindesine (Desacetylvinblastine Amide)

Vindesine has been investigated for antibody-mediated targeting by Rowland and co-workers.[428-432] Rowland has pointed out that effective clinical dosages of vindesine are 10 to 100 times lower than other drugs that have been linked to antibodies, e.g., the potency is intermediate between other anticancer drugs and toxins.[428] Vindesine also has a phase-specific antimitotic effect, whereas toxins linked to antibodies could affect both dividing and nondividing cells that took up conjugate nonspecifically.[428,431] Vinblastine derivatives have a carboxy function (C23) which can be variously substituted without loss of cytotoxicity.[433] For example, desacetylvinblastine possesses a methyl ester while vindesine has an amide. The most common conjugation procedure has been to first react desacetylvinblastine with nitrous acid.[434] The resulting azide need not be isolated, but can be reacted directly with the Ig at pH 9.[428,433] The site of linkage has not been identified but is presumed to be the epsilon amino group of lysine.[433] Incorporation ratios of 4 to 11 mol/mol of IgG have been reported.[429,432] Conjugates prepared with mouse monoclonal antiosteogenic sarcoma IgG2b were shown to retain essentially full antibody activity based on a competitive binding assay.[431] Conjugates prepared with a polyclonal sheep anti-CEA preparation were observed to localize on target cells.[430]

Vindesine-antibody conjugates have been evaluated by an in vitro indirect targeting assay using tumor cells coated with an appropriate rabbit antibody.[428] The vindesine-antirabbit-Ig conjugate produced significant inhibition of antibody-coated cells but not uncoated cells. In another in vitro assay involving inhibition of incorporation of ^{75}Se-methionine into osteogenic sarcoma cells, target-specific inhibition could not be demonstrated. However, after pulse exposure, the conjugated drug was less cytotoxic by several orders of magnitude than the free drug but selective toward target cells.[429,431] When vindesine was conjugated to a polyclonal sheep anti-CEA antibody, exposure of CEA-bearing CALU-6 cells showed that the conjugate was considerably more potent than free vindesine, a mixture of vindesine and antibody, or a conjugate synthesized with normal sheep IgG.[428] Evidence for localization of the specific conjugates in sarcoma-bearing nude mice was obtained by using a radioiodinated conjugate. The tissue-

to-blood ratio was of the order of threefold greater for tumor tissue than for any other tissue. In other experiments, it was shown that the cytotoxicity of vindesine conjugated to a monoclonal anti-p97 antibody toward different melanoma cell lines varied directly with the surface density of p97 antigen. Repeated injections (ten during 34 days) of two different conjugates with monoclonal anti-CEA antibodies (an IgG2a and an IgG1, respectively) were administered to human colorectal carcinoma-bearing nude mice. There was substantial suppression of tumor growth compared to controls up to approximately 60 days with both conjugates, after which tumor growth accelerated in the animals given the IgG2a conjugate. In contrast, the IgG1 conjugate suppressed tumor growth until 90 days, the reported duration of the experiment.[429] Ford et al.[432] were able to show that a radiolabeled conjugate of vindesine and a polyclonal sheep anti-CEA antibody localized in five out of eight patients with advanced colorectal and ovarian carcinoma. No measurements of antitumor effects were reported.

5. Nucleosides

Hurwitz et al.[435] have investigated two nucleotide analogs, ARA-C and FUR, which are competitive inhibitors of DNA synthetic enyzmes. Both possess vicinal hydroxyl groups in their sugar moieties, and cytosine has an amino group as a potential linkage site. Modification of either of these groups might be expected to affect the interaction with target molecules in vivo but the amino group may be more critical. Linkage of ARA-C to an affinity-purified goat antibody against surface IgM on 38C leukemia cells was carried out by reacting polyaldehyde dextran with the drug and then with the IgM. Finally, sodium cyanoborohydride was used to stabilize the linkage. A total of 25 to 60 mol of tritium-labeled drug was bound per mole of IgM with almost full retention of antibody activity. The antitumor effect was assayed in vitro by enumeration of viable 38C cells or by inhibition of incorporation of [methyl-^{3}H]-thymidine and/or -uridine after pulse exposure to test agent for either 2 or 24 hr. It was found that the drug-dextran conjugate and drug-dextran-antibody conjugate were somewhat more effective than the free drug at certain concentration levels. Some specificity toward target tumor cells could also be seen with the antibody conjugate.

The linkage methodology for conjugation of FUR also used dextran as intermediary, but in this case the nucleoside sugar residue was oxidized with periodate rather than the dextran. This produced two vicinal aldehyde groups capable of reacting with the hydrazide derivative of dextran. The binary hydrazone conjugate was linked to the IgM using glutaraldehyde and stabilization by reduction was not carried out. There was incorporation of 7 to 24 mol of tritium-labeled FUR per mole of Ig with retention of most of the antibody activity, although less than in the ARA-C conjugate. Using the same in vitro assays as with ARA-C conjugates, it was found that at high drug concentrations (1 μg/mℓ) there was no marked difference in the inhibitory effects of the free drug and the binary and ternary conjugates. The inhibitory effects of conjugates were compared to fluorouracil rather than FUR since the sugar ring in effect had been destroyed by oxidation. Thus, one could postulate that the base rather than the nucleoside would be released from the conjugate intracellularly and converted both to FUMP, which is incorporated into RNA, and to FUdMP, which inhibits thymidylate synthetase. However, these authors pointed out that it is possible that the conjugates could operate via a mechanism different from that of the free drug.

6. Bleomycin

Bleomycin has been linked to a murine anti-HLA IgG1 MAB by Manabe et al.[358] Polyaldehyde dextran was first incubated with bleomycin and then with the IgG followed by borohydride treatment. A total of 58 mol of drug was incorporated per mole of IgG. Assay of the activity of the agent by growth inhibition of *Mycobacterium*

smegmatis gave an apparent retention of 18% of drug activity. There was complete retention of antibody activity when measured by membrane immunofluorescence. The ternary bleomycin conjugate was 15-fold more toxic to cells possessing HLA than was free bleomycin but significantly less toxic than free bleomycin toward cells lacking target HLA.

7. Mitomycin C

Mitomycin C has been studied by two groups of investigators who took distinctly different approaches to conjugation.[336,359] Kato et al.[336] coupled this agent to a polyclonal horse anti-AFP Ig at the N-1a position by using a glutaric acid-based spacer arm. A 4-carboxybutyryl substituent was attached to the mitomycin C by reaction with glutaric anhydride. The corresponding active ester was then synthesized by reaction with NHS and dicyclohexylcarbodiimide. Coupling with the Ig was effected with a 20-fold molar excess of the drug derivative to achieve an incorporation ratio of 8 mol of drug per mole of Ig. This method avoids Ig polymerization and allowed higher protein recoveries and higher incorporation ratios than a previously used cyanogen bromide method.[436] The aziridyl amide linkage was slowly hydrolyzed with a half-life of 2.6 days at pH 7.4 and 0.4 days at pH 4, which may facilitate lysosomal breakdown. A fivefold increase in cytotoxicity in vitro toward target AH66 hepatoma cells was achieved with the specific conjugate in comparison with a mixture of free drug and antibody. A nonspecific conjugate was approximately as inhibitory as the free drug. Multiple i.p. injections over a 10-day period of the specific conjugate to i.p. tumor-inoculated rats were able to prolong mean survival from 17 days (saline controls) to 57 days. Mean survival was 23 days for the free drug group and 28 days for the nonspecific conjugate group. In the second approach, Manabe et al.[359] used dextran to prepare a conjugate with monoclonal anti-H-1 IgG1 antibody in essentially the same way as described for MTX,[360] etc. except that the reaction order was Ig followed by mitomycin C. The incorporation was 88 mol of drug per mole of IgG. Based on an assay of growth inhibition of *Escherichia coli*, conjugated mitomycin C was found to be 2.4% as effective as the free antibiotic. Membrane immunofluorescence of target tumor cells revealed substantial retention of activity in the conjugates and the conjugate was tenfold more cytotoxic toward target cells in vitro than the free agent.

8. Neocarzinostatin

Neocarzinostatin consists of an acidic protein complexed to a cytotoxic chromophore producing DNA damage. Kimura et al.[437,438] have conjugated this antibiotic protein to a rabbit IgG against a human leukemia cell line (NALL-1) using ECDI. Indirect membrane immunofluorescence (FITC-labeled goat antirabbit IgG) showed that antibody activity was retained. The specific conjugate inhibited growth of NALL-1 cells to the same extent as free neocarzinostatin in a continuous exposure assay but, after pulse exposure, to a greater extent than neocarzinostatin alone or neocarzinostatin conjugated to normal rabbit IgG. Inhibition of tritiated thymidine deoxyriboside uptake by antibody-linked neocarzinostatin was greater than by free neocarzinostatin or antibody or by neocarzinostatin plus antibody unlinked. The order of effectiveness after i.p. administration of agents to immunosuppressed Syrian hamsters inoculated intraperitoneally with 5×10^6 BALL-1 cells was specific conjugate > neocarzinostatin linked to NRG or neocarzinostatin plus antibody or neocarzinostatin > antibody or saline. If tumors were inoculated subcutaneously, the effectiveness of the specific conjugate was no greater than neocarzinostatin alone, neocarzinostatin plus antibody, or neocarzinostatin linked to normal rabbit IgG. Luders and co-workers used SPDP to effect a disulfide linkage between the apoprotein of neocarzinostatin and an IgG1 MAB against a high molecular weight antigen (A-1-43) on the human melanoma cell line A-375.[438a]

The ternary conjugate was purified with Protein A Sepharose and then the biologically active chromophore of neocarzinostatin was added in excess to form the specific drug complex. In this method, the apoenzyme is essentially being used as an intermediary. The specific conjugate was 100-fold more toxic toward A-375 cells, as measured by tritiated-thymidine uptake, than free neocarzinostatin or neocarzinostatin conjugated to normal mouse IgG1. There was also a selectivity factor of 40 to 50 observed in comparing the action of the specific conjugate on antigen-positive and antigen-negative cells.

B. Antibody-Bound Toxins

Considerable interest has developed in the use of catalytically active protein toxins for conjugate preparation. In the category of catalytic agents, one could include essentially any enzyme capable of disrupting membrane constituents (e.g., phospholipase-C[14]), intracellular DNA or RNA (e.g., ribonuclease), or various intracellular proteins. However, the emphasis has been on the group of bacteria- and plant-derived polypeptide/protein agents categorized as toxins. Toxins that have been conjugated to antibodies include gelonin, pokeweed antiviral protein, abrin, ricin, diphtheria toxin, and *Pseudomonas* exotoxin. There are two main types: single-chain toxins and linked polypeptides. The linked polypeptides consist of a A chain which is the cytotoxic entity and a B chain which is a lectin responsible for binding to cell surface carbohydrate moieties. For example, the B chain of ricin binds at cell surface galactose-containing moieties and also plays a role in transfer of the A chain to the cytoplasm. If the B chain is removed by cleavage of the disulfide linkage, then the isolated A chain is likely to exhibit cytotoxicity toward whole cells that is of the order of 5 to 6 logs less than the intact toxin.[439,440] However, the isolated A chain retains its ability to inhibit protein synthesis as measured in a cell-free system. The literature has been reviewed in recent years.[14,18,29,340,344,362,364-366,440-443] Also, the authors have considered some of the special features of linking a polypeptide or protein to an antibody in Section IV on methods of linkage.

In general, there are two major modes of action by which agents exert their cytotoxic effect. An agent can either interact stoichiometrically with its target molecule and block the target molecule's normal function or it can enzymatically modify the target molecule so that it is rendered nonfunctional. If action is stoichiometric, then a sufficient concentration of the agent must be attained to incapacitate a critical proportion of the target molecules. Incapacitation will depend on the relative concentrations of agent and target molecule and the equilibrium constant governing the interaction between them. The interaction between MTX and DHFR is illustrative. MTX binds extremely tightly to this enzyme and thus inactivates it. However, the usual intracellular level of DHFR apparently exceeds that required for cell viability by a substantial margin so the required extent of inactivation is high. It has been shown that the intracellular concentration of MTX must exceed that of DHFR to be cytotoxic.[407] Furthermore, the fraction inactivated may drop below the critical value during therapy because of increases in the intracellular level of DHFR or decreases in its affinity for MTX.

Agents that exert their effect by catalyzing an inactivating reaction can be cytotoxic at very low concentrations. They can in principle continue to act on newly synthesized target molecules as long as they themselves are not inactivated, e.g., by intracellular hydrolases. It has been estimated for at least certain toxins that as little as one molecule per cell may be sufficient for exerting a cytotoxic effect.[444,445] This very high potency renders the requirement for specificity in the carrier even more stringent and requires that the purification procedure applied to a conjugate eliminate, to the greatest extent possible, residual contamination by unreacted whole toxin molecules. In the case of antibodies, this implies low cross-reactivity toward antigens on normal cells, low levels

of the tumor antigen marker in normal tissues, and low uptake by phagocytic cells. Since the targets of protein toxins are intracellular, endocytosis of conjugated toxin or its A chain is essential. As with agents in general, an ineffective conjugate will result if the carrier antibody has low affinity or the complex with the target antigen is not easily endocytosed because of size or other factors relating to the nature of the antibody or its target antigens. Theoretically, for specific targeting, it is tempting to remove the toxin B chains that bind to a wide variety of cells so that the specificity of the carrier antibody selectively targets the A chain. However, there is a wide variation in the uptake of A chain conjugates. The B chain seems to augment the internalization of conjugates. For example, it has been observed that the presence of the ricin B chain in conjugates seems to facilitate action of the A chain on target cells even when direct B-chain binding to cell membrane receptors is blocked by lactose.[446-448] Nevertheless, active A-chain conjugates have been obtained.

For designing effective whole toxin conjugates, it is desirable to avoid intra- and intermolecular cross-linkage that can result in loss of antibody and toxin activities, e.g., through blockage of the respective combining or active sites, through covalent linkage between the A and B chains of the toxin, etc.[449] A common choice of linkage group is the disulfide bridge, and its utility for this purpose is illustrated by comparative studies with the linked polypeptide toxin, abrin, conjugated to antimouse lymphocyte IgG by two different methods.[449] SPDP was used to produce a disulfide bridge conjugation and the NHS ester of chlorambucil to produce an amide and pipirazine ring conjugation.[364] The incorporation ratio in this application was close to one. Both these reagents have been described in considering methods of linkage. Abrin conjugated by either method was an equally effective inhibitor of protein synthesis in a cell-free system. However, spleen cells in culture were twice as susceptible to the disulfide-linked conjugate, suggesting relatively easier intracellular release of an active A-chain moiety itself through disulfide interchange. Abrin linked by a pipirazine group involving sites on the A chain of the toxin would presumably be more stable.[449]

The role of the specific carrier in antibody-mediated targeting of toxins is illustrated by the studies of Ross et al. with diphtheria toxin.[450] Daudi cells are relatively insensitive to this toxin since 1 $\mu g/m\ell$ does not inhibit protein synthesis in cells in vitro. Antilymphocyte IgG-whole diphtheria toxin conjugates were prepared by a chlorambucil-mixed anhydride procedure. This conjugate was shown to be distinctly cytotoxic toward the Daudi cells; a concentration of 0.5 $ng/m\ell$ inhibited tritiated leucine uptake by 50%. The same behavior was observed when the $F(ab')_2$ fragment of this antibody was used for conjugation. Pretreatment of cells with free antibody or with diphtheria antitoxin lowered inhibition and a conjugate prepared with a nonspecific IgG was ineffective. These findings could not be duplicated with mouse spleen cells and a corresponding conjugate with antimouse lymphocyte antibodies. More recently, Pirker et al.[8] have demonstrated that the in vitro toxicity of an antitransferrin receptor MAB-*Pseudomonas* exotoxin conjugate to several human ovarian cancer cell lines was directly related to the extent of binding and internalization of the conjugate. Verpamil enhanced the toxicity of this conjugate.

Antibody conjugates synthesized with whole toxins suffer from the disadvantage that the B chain in the conjugate may still promote uptake by nontarget cells, resulting in high host toxicity. In one approach to minimize nonspecific binding of the B chain, intact ricin was coupled via iodoacetylated whole toxin which produces thioether-linked conjugates characterized by lowered galactose recognition.[367] (This lack of cytotoxicity to nontarget cells does not apply to iodoacetylated whole ricin itself.) B-chain-recognition-based uptake by nontarget cells can also be blocked in vitro by high concentrations of galactose or lactose in the incubation medium to saturate binding sites for nonantibody-mediated uptake of abrin or ricin conjugates. However, a further

difficulty can arise from the fact that mannose-containing oligosaccharide substituents on antibody-conjugated toxins can promote uptake by phagocytic cells via mannose receptors.[451]

Other approaches for eliminating nonspecific binding via B chains, especially in vivo, include removal of the B chain prior to conjugation or the use of single-chain toxins (so-called hemitoxins). A useful attribute of single-chain toxins from the point of view of laboratory manipulation is that they are not toxic until conjugated to a carrier that promotes endocytosis. An example is saporin, a single-chain toxin from *Saponaria officinalis* which has been conjugated, by using SPDP, to an anti-Thy 1.1 MAB (OX7) and its $F(ab)_2$ fragment.[451a] The purified conjugate fraction used for biological testing consisted chiefly of 1:1 and 1:2 (toxin to MAB) species. The conjugated toxin did not inhibit protein synthesis in a reticulocyte lysate, indicating that the toxin must be released from the carrier protein for manifestation of cytotoxicity. The specific whole IgG or $F(ab)_2$ conjugates were strongly inhibitory toward pulse-exposed Thy 1.1-positive cells in culture (but not toward Thy 1.1-negative cells). I_{50} values for inhibition of tritiated leucine uptake ranged from 3×10^{-12} *M* to 1×10^{-10} *M*. The pattern of response to Concanavalin A (Con A) and *E. coli* lipopolysaccharide mitogens by spleen cells exposed to the saporin-MAB conjugate suggested that T-lymphocytes (which express Thy 1.1 antigen) were selectively killed. Measurement of acute toxicity toward mice indicated that conjugation of saporin to IgG increased toxicity by a factor of 8 to 16. Mice inoculated intraperitoneally with 10^6 AKR-A tumor cells and then given one i.v. injection of the specific conjugate after 24 hr, survived an average of 30 days longer than untreated control mice. Three out of eight animals remained free of ascites tumor during the experiment. (Two of these animals developed solid s.c. tumors at the site of i.p. inoculation, suggestive of relative inaccessibility.) The specific $F(ab)_2$ conjugate was substantially less effective than the IgG conjugate. Antibody alone, toxin alone, antibody plus toxin unlinked, and toxin linked to an irrelevant antibody did not prolong survival. Calibration experiments showed that the pattern of survival of treated mice given 10^6 tumor cells was similar to that of untreated mice given 10 tumor cells. This finding was interpreted to indicate 99.999% elimination of tumor cells after inoculation. Tumor cells isolated from ascites tumors that grew in conjugate-treated animals had the same sensitivity in vitro to the specific conjugate and so did not represent a resistant subclone. The corresponding anti-Thy 1.1 MAB conjugate of ricin A chain had cytotoxicity in vitro that was comparable to that of the specific saporin conjugate, but the former produced survival in vivo for tumor-inoculated mice that was equivalent to only 99% killing of tumor cells. These authors suggested that the carbohydrate moiety present on ricin A chain but not saporin may lead to clearance by the reticuloendothelial system. The linkage in the conjugate of positively charged saporin may also be more stable during transit in vivo than that in the conjugate of uncharged ricin.

Another example of a single-chain toxin is pokeweed antiviral protein. Conjugation to antibodies is reported to confer on it cytotoxicity toward eukaryotic systems that is of the same order as ricin.[452] Like ricin, the mode of action involves interference with the function of protein synthesis elongation factors.[452] Recent studies show the potential usefulness of toxin conjugates in selective elimination of human cancer cells. Uckun et al.[373,453] linked pokeweed antiviral protein to an IgG_1 MAB against human B- and pre-B-cells by a disulfide interchange method. The ratio of toxin to IgG was 2:1 as shown by RIA for the toxin polypeptide. This conjugate at a toxin concentration of 5 μg/mℓ produced 80% inhibition of tritiated leucine uptake by target B-ALL cells but did not affect nontarget normal bone marrow cells. A nonspecific conjugate was without effect on the B-ALL cells. A clonogenic assay showed that almost 6 logs of killing of target lymphoma cells could be achieved in the presence of chloroquine, which potentiated cytotoxicity. Moreover, this effect was achieved under conditions where there

was a 100-fold excess of normal marrow cells. There was less than a 50% loss of pluripotent stem cells.

Most conjugates containing cleaved A chains have been synthesized by disulfide linkage utilizing the A chain -SH formed by reductive splitting of the original disulfide bridge between the A and B chains of the parent toxin, a procedure which is generally capable of substantially retaining antibody activity. This has been discussed in dealing with methods of linkage. In effect, the method preserves the original linkage group but without preserving the ancillary role(s) of the B chain in toxin transport and action as stated above. Experiments involving reductive cleavage of conjugates and isolation of released A chains allowed the demonstration that the released A chains were fully inhibitory in a cell-free protein synthesis system if the original conjugation antibody was of the IgG class.[366] However, not all chains were fully active if the original conjugation antibody was of the IgM class. An interesting comparison of linkage groups is that between disulfide and thioether bridges. One way of linking via a thioether bridge is with 6-maleimidocaproic acid to introduce a 9-atom spacer. Ricin A chain linked in this way had 30% activity in a cell-free system, and the greater stability of the thioether bond indicates that this activity is indeed due to the bound A chain and appears to reflect some steric hindrance.[366] These thioether-linked conjugates were only 1% as active as disulfide conjugates on intact cells, indicative of a failure of transport. This suggests that conjugate action involves the splitting of the toxin chain. However, introducing a longer disulfide-containing spacer, which hypothetically might be split more readily, did not improve cytotoxicity against intact cells, although activity in a cell-free system was greater.[366]

Conjugates of ricin A chain have also been prepared using the biotin-avidin system outlined under Section IV, Methods of Linkage.[342] Ricin A chain was coupled to PDT-avidin by disulfide interchange and biotin to Ig by an active ester method. The ricin incorporation assayed by inhibition of protein synthesis using a rabbit reticulocyte lysate was a little over 1 mol/mol of avidin. When BALBc spleen cells were exposed to biotinylated-antimouse Ig followed by ricin-A-chain-substituted avidin, the subsequent lipopolysaccharide response was poor and the Con A response was good. When the first exposure was to biotinylated-anti-Thy-1.2, the converse responses were observed. These results indicated specific cytotoxicity toward target cells. Treatment of cells with either binary conjugate alone did not affect responsiveness. These investigators also used the $F(ab')_2$ fragment of a polyclonal IgG against the putative T-cell replacing-factor (TRF) acceptor sites to prepare a conjugate that was capable of selectively eliminating a B-cell subset having these acceptor sites. Advantages of the sequential exposure approach were stated to be the convenience of having available supplies of toxin-substituted avidin and the potential for binding multiple ricin A chains to Ig that is substituted with multiple biotin molecules. If the sequential use of biotin-Ig and ricin-avidin were tried in vivo, the possibility arises that ricin-avidin could combine with biotin of the host, thus eliminating targeting specificity.

Another related system that might allow for in vivo targeting is that consisting of the ribonuclease-S peptide and ribonuclease-S protein. The affinity constant governing this interaction is not as large as the constant for biotin-avidin, but it is still in the range of antigen-antibody constants, i.e., 10×10^9 [454] and the coupling chemistry should not be difficult, particularly since ribonuclease is a small, stable protein. (Either of these noncovalent-binding systems ought to be feasible for preparing ternary conjugates intended for administration as such.)

Conjugates that are prepared by the linkage of the cleaved A chain of protein toxins that possess both A and B chains are generally less toxic than whole toxin conjugates by a couple of orders of magnitude. Raso et al.[455] used hybrid antibodies with one specificity for target cells and the other for ricin A chain to deliver either whole toxin

or A chain to Daudi cells. The I_{50} of the cleaved ricin A chain conjugate was 10- to 100-fold greater than that of the whole toxin conjugates, which suggests augmentation of toxicity by the B chain. Furthermore, conjugates containing cleaved A chains exhibit variable cytotoxicity toward target cells in vitro, high in the case of A chains of several toxins conjugated to antibodies directed against the transferrin receptor,[368] low in the case of diphtheria toxin or ricin A chain conjugated to anti-Thy 1.1. or W 3/25 IgG.[364] The variable properties of A chain immunotoxins are further illustrated by experiments comparing diphtheria toxin A chain and ricin A chain conjugated by a disulfide linkage to epidermal growth factor which showed that the ricin conjugate was cytotoxic toward 3T3 cells at 0.01 to 1.0 n*M*, whereas the diphtheria toxin conjugate was not cytotoxic at concentrations of the order of 30 n*M*.[7] Diphtheria toxin A chain conjugated to human placental lactogen by use of methyl-5-bromovalerimidate was also inactive against mammary gland explants, although it retained ADP-ribosyltransferase activity and the conjugate could bind to lactogenic receptors.[5,456] Nevertheless, some of the A chain conjugates have been found to be effectively tumor inhibitory in vitro as well as in tumor-bearing animals. For example, a disulfide-linked conjugate between diphtheria toxin A chain and a monoclonal IgG1 against the guinea pig L10 hepatocarcinoma has been prepared by Bernhard et al.[457] The purification procedure took advantage of the binding affinity between NAD^+ and diphtheria toxin A chain. The reaction mixture was passed through an NAD-Sepharose column which adsorbed the IgG-linked toxin and eliminated unreacted IgG. Subsequent passage through a Sephadex® G-200 column eliminated unreacted A chain. The purified conjugate bound to L10 cells and not L1 cells in vitro. L10 cells in culture were reported to be 100% killed by a 24-hr pulse with conjugated A chain at 100 n*M*, whereas antibody alone was not cytotoxic. Prior incubation with antibody alone at 1 μM completely prevented cell killing by the conjugate. The anti-L10 cell IgG1 was shown to localize in tumors in vivo,[458] so the antitumor effect in vivo was assessed by treating intradermally tumor-inoculated guinea pigs with conjugate intravenously on day 2 or on day 7 after inoculation. Tumor growth was slightly inhibited by the treatment on day 2 but substantially retarded by the treatment on day 7. In the latter case, tumor size regressed and did not increase significantly until day 17, after which growth occurred at a rate approximately parallel to the controls, i.e., the single immunotoxin treatment retarded the onset of growth rather than decreasing the rate of growth. Ghose et al. obtained somewhat similar results in treating melanoma-bearing mice with an MTX-antimelanoma antibody conjugate.[302] In another investigation, a mouse BCL_1 model was adapted to mimic a clinical situation in which patients have a large tumor burden.[459] Affinity-purified ricin A chain was conjugated to the PDT derivative of an anti-idiotypic antibody by disulfide interchange. Purification of the resulting conjugate was by gel filtration and affinity chromatography. The antibody-linked toxin treatment was instituted after reduction of tumor bulk by irradiation and splenectomy. Under these conditions (but not with irradiation alone), it was possible to achieve remission in treated animals, whereas control animals only survived for 7 days. Blood from mice apparently in remission did not produce leukemia when given to normal mice and so did not contain tumor cells. However, blood from long-term survivors was capable of producing tumors under these conditions, indicating that tumor cells persisted in treated animals. It was pointed out that successful immunochemotherapy was dependent on preliminary cytoreduction, given present limits on therapeutic indexes for antibody-linked toxins.

A variety of approaches have been taken to augment the cytotoxicity of A chain toxin conjugates. These include simultaneous exposure to lysosomotropic agents, viruses, or cleaved B chains. The behavior of chloroquine and ammonium chloride in potentiating cytotoxicity of pokeweed antiviral protein and ricin A chain conjugates has already been alluded to. Akiyama et al.[460] have recently reported on the potentia-

tion of cytotoxic activity of *Pseudomonas* exotoxin-antibody conjugates by calcium channel blockers and a lysosomotropic agent. Conjugates were synthesized by the same method and with the same antitransferrin receptor antibody (HB21) as used by FitzGerald et al.[461] (see later) and with EGF. Conditions were chosen so as to give 1:1 conjugates. A colony inhibition assay with KB cells showed that verapamil, D-600, diltiazem, and the lysosomotropic agnet, beta-glycylphenylnaphthylamide, increased cytotoxicity of conjugates in a concentration-dependent manner. For example, D-600 at 20 $\mu g/m\ell$ gave the largest effect, lowering the I_{50} for inhibition of cell growth from 3 to 0.2 $ng/m\ell$. These agents alone had no effect on colony formation at the concentrations used. Potentiation effects were usually less on other cell lines. Another assay based on inhibition of protein synthesis measured by tritiated leucine uptake gave results in which the conjugates exhibited less cytotoxicity, but there was greater potentiation by agents. The mechanism of potentiation is not clear. In a previous study by this group,[462] verapamil-treated KB cells delayed lysosomal degradation of ^{125}I-labeled EGF, indicating interference with lysosomal function as an underlying mechanism. Also, hydrolysis of beta-glycylphenylnaphthylamide in lysosomes has been reported to produce a metabolite that damages lysosomal and perhaps other cell membranes.

FitzGerald et al.[461,463] have investigated the ability of human adenovirus type 2 to increase the toxicity of *Pseudomonas* exotoxin conjugated to monoclonal antitransferrin receptor antibodies or EGF. Conjugation was achieved by disulfide interchange.[464] Methyl-4-mercaptobutyrimidate was used to introduce thiol groups into both toxin and carrier protein (an average of two in the toxin and one in the carrier). The modified toxin was treated with 5,5′-dithio-*bis*-nitrobenzoate to produce a thionitrobenzoate derivative, after which conjugation to thiolated carrier protein was carried out by incubation with a threefold molar excess of the thionitrobenzoate derivative. The conjugate was isolated by adsorption on protein A at pH 8 followed by elution at pH 6. ADP ribosylating activity was retained in the conjugate. Exposure of cells in culture followed by measurement of inhibition of tritiated leucine uptake showed that the antibody conjugates had I_{50} values of the order of 0.1 to 0.3 n*M*, whereas the cysteine-substituted toxin was not cytotoxic at 10 n*M*. This control was included because reaction of the toxin with methyl-4-mercaptobutyrimidate abolishes binding to the receptor. This effect on binding indicates that uptake by nontarget cells would not occur if conjugate disulfide linkages were reduced in vivo.[461] Concurrent exposure of KB cell monolayers to conjugate plus adenovirus increased toxicity 100- to 300-fold. Adenovirus alone did not inhibit protein synthesis under the experimental conditions. The cytotoxicity of conjugates depended on the human cell line tested. Earlier studies by Trowbridge and Domingo[465] had shown that ricin A chain conjugated to antitransferrin receptor antibodies was cytotoxic, and FitzGerald et al.[461] found that adenovirus was also able to potentiate the effect on protein synthesis of their ricin A chain-antibody conjugates. It was concluded from immunofluorescence measurements that both conjugate and adenovirus were taken up into the same receptosome, allowing the virus to disrupt the receptosome and so increase release of toxin molecules into the cytosol. A capsid protein of the virus appears to mediate the effect, so the isolated protein might be utilized in combination with antibody conjugates to increase therapeutic effectiveness.

The cytotoxicity of A chain conjugates has been restored by inclusion of free B chains in an in vitro protein synthesis assay system.[374,451,466] The effect was about fivefold and is thought to involve increased efficiency of entry into the cytoplasm rather than increased binding of conjugated A chain.[466,467] Vitetta et al.[374,451] have investigated potentiation of the cytotoxicity of ricin A chain-IgG-antibody conjugates by ricin B-chain-IgG-antibody conjugates, both linked by disulfide interchange. They used affinity-purified rabbit IgG against human Ig since their test system consisted of a target

Burkitt's lymphoma cell line possessing surface IgG. The ternary conjugates were purified by Sephacryl S-200 chromatography and then by affinity chromatography with immobilized human Ig to eliminate residual free A or B chains. Solid phase RIA using ^{125}I-labeled anti-A or -B indicated one to two A or B chains per Ig. In vitro measurements of uptake of tritiated leucine by Daudi cells showed that little toxicity was manifested separately with the A-chain conjugate at 0.3 μg/10^5 cells or with the B-chain conjugate at levels substantially above this. In contrast, mixtures of the two gave significant toxicity. The effect reached almost an order of magnitude with a mixture at 0.3 (A conjugate) and 2.6 (B conjugate) μg/mℓ. Synergism with mixtures of free chains and either of the conjugates was found only at high concentrations and was reported to be modest. A and B chains conjugated to irrelevant IgG gave no synergism, with or without galactose. This is indicative of a decrease in B chain-based binding on its conjugation to an Ig. This approach shows that it may be possible to circumvent the nonspecific toxicity associated with whole linked-polypeptide-toxin conjugates without losing the B chain function.

One variation in conjugation is to link the A chain to a univalent F(ab) antibody directed against the target cell surface and the B chain to an anti-Ig antibody.[451] The use of a F(ab) fragment was expected to avoid binding to Fc receptors on nontarget cells and lead to delayed endocytotic uptake, thus allowing the B-chain conjugate to be administered at a later time. The same affinity-purified rabbit antihuman Ig as described above was used as the carrier for the ricin A chain and goat antirabbit Ig antibody was used for the B chain. Incorporations of one to three A or B chains per antibody molecule were obtained with less than 1% contamination by free chains. Cytotoxicity was assayed using sequential 15-min incubations with conjugates followed by 22 hr of growth in conjugate-free medium, after which uptake of tritiated leucine was determined. The B chain linked to antirabbit Ig potentiated cytotoxicity of the cell-surface targeted A chain conjugate in a manner comparable to that observed before with both chains linked to the same antisurface Ig carrier antibody. Synergism was not observed if the B chain was linked to an irrelevant Ig. The conjugate of F(ab) and A chain was equal in cytotoxicity to the corresponding whole Ig conjugate and was also potentiated by B chain linked to antirabbit Ig. The synergistic effect decreased linearly with increase in the time interval between exposure to the A-chain and B-chain conjugates, e.g., half the effect was still present at 5 hr. The authors suggest that in an in vivo context targeted A-chain univalent F(ab) conjugate which did not bind to target cells could be substantially eliminated prior to administration of a B-chain conjugate targeted to the A-chain carrier antibody.

C. Antibody-Bound Radionuclides

Radioisotopes have several characteristics that make them attractive for antibody-mediated targeting against tumor cells. Ionizing radiations are widely used in the treatment of cancer, and factors determining the response of mammalian cells to different types of radiation are fairly well known. Cell damage is caused either by emitted charged particles or quanta of energy and therefore there is no necessity for the isotope to be endocytosed. Binding of the isotope-carrying antibody can deliver radiation to several adjacent cells that may lack the target antigen.[296]

The criteria of suitability of antibody-linked radioisotopes for therapy differ from those for imaging. A high tumor-to-normal tissue ratio of localization is the most important factor in tumor imaging. Furthermore, for conventional external imaging (i.e., methods not based on positron emission), the radioisotopes must be gamma emitters with a short half-life and should be rapidly cleared from the body to reduce radiation hazards. For therapy, high and prolonged concentration of the radioisotope at the tumor site are desirable features and a relatively low tumor-to-normal tissue ratio of

localization may be adequate. Radioisotopes for therapeutic purposes should produce a tumoricidal amount of ionization at the site and the radioactivity should be adequately localized, have low penetration to avoid systemic effects, and remain tumor bound for a period sufficient to deliver a tumoricidal dose. The optimal time to deliver such a dose will depend upon the decay scheme of a given radioisotope. Eckelman et al.[468] have listed the physical properties and pharmacokinetics of some radioisotopes that may be of use in antibody-mediated tumor imaging and therapy.

^{131}I is the most used radionuclide for antibody-targeted therapy of experimental and human cancer.[143,308,469-473] Definite evidence of tumor regression has been obtained only with this radioisotope.[310,469,470] Reasons for its employment in radioimmunotherapy include its established use in the treatment of hyperthyroidism, the availability of several mild methods for iodination of Ig, its physical characteristics, and the range of radiation in tissues. The reaction conditions for iodination of proteins can be manipulated[468] so that it is possible to control the level of incorporation of the isotope.[15] Substantial antibody activity is retained when the level of incorporation of ^{131}I does not exceed two atoms of iodine per molecule of IgG.[300,310,469,474] Higher levels of incorporation led to a decrease in the affinity of the antibody and its faster clearance from serum.[310] The physical characteristics of ^{131}I are such that, in spite of the limited cell surface concentration of TAA, antibodies labeled with less than two atoms of ^{131}I per IgG molecule can deliver a tumoricidal amount of radioactivity.[12,310] The effective beta radiation from ^{131}I has a range in tissue corresponding to several times the diameter of a cell.[296] Although this is likely to eradicate adjacent tumor cells that lack the target antigen and do not bind the labeled antibody, adjacent normal cells can also be damaged. In any case, nonuniform distribution of radioactivity may fail to deliver lethal amounts of radioactivity to all adjacent cells. The features of ^{131}I that limit its usefulness for antitumor targeting include the susceptibility of antibody-bound iodine to dehalogenation in vivo[468] associated with uptake by unblocked thyroid, immuno- and myelosuppressive effects,[310,469] potential carcinogenic effects (not so far observed in adults treated with ^{131}I for hyperthyroidism),[475] and the very rare incidence of allergy to iodine.

Use of radionuclides that emit softer β-rays than ^{131}I or of alpha emitters may achieve more precise and uniform cell kill, but the effective range does not exceed that of a single diameter and adjacent antigen-negative tumor cells are not likely to be affected. Nevertheless, ^{211}At appears to have several desirable features for use in immunoradiotherapy of cancer. The alpha particles emitted during its decay have high average energy (Ea = 6.8 MeV), high linear energy transfer (i.e., induce high specific ionization), and an effective range of several cell diameters. Their effect on cells and tissue is fairly independent of oxygen saturation, and tumoricidal effects in vivo have already been demonstrated.[476] In another approach to immunoradiotherapy of cancer, ^{10}B has been linked to anti-TAA antibodies,[477,478] with the expectation that after exposure to slow neutron radiation thermal neutrons will be captured with the release of 2.79 MeV of energy. However, tissue penetration of thermal neutrons is poor and whether the ^{10}B really adds to the effect of neutron beams alone is still controversial.[479,480]

VI. POTENTIAL APPLICATIONS AND CLINICAL EXPERIENCE

Drug-antibody conjugates and radiolabeled antibodies have been used in preliminary phase I investigations in cancer patients. In general, drug conjugates have shown a more consistent antitumor activity than immunotoxins in tumor-bearing animals. Furthermore, the mode of action and pharmacokinetics of cancer chemotherapeutic agents

and radionuclides are better understood than those of the protein toxins. Finally, as cancer chemotherapeutic agents are widely used clinically, the use of their conjugates entails fewer ethical problems than immunotoxins. This has confined the use of immunotoxins mainly to the elimination of target cells, e.g., leukemia/lymphoma cells or subsets of immunocompetent cells, from bone marrow in vitro prior to bone marrow transplantation.

A. Purging of Neoplastic Cells In Vitro

Autologous bone marrow transplantation is being increasingly used in the management of leukemias and non-Hodgkin's lymphomas.[481,482] Marrow obtained from a patient, even in remission, is likely to contain small numbers of neoplastic cells which may cause relapse of the disease when the marrow is transfused back after supralethal chemoradiotherapy. Selective and complete elimination of contaminating malignant cells from the bone marrow to be retransfused thus constitutes a major problem. Methods that have been used to remove tumor cells from marrow include physical separation,[254] cytotoxic drugs,[483] anti-TAA antibodies together with complement,[118,484-487] MAB linked to intact ricin,[363,378,488] MAB linked to ricin A chain,[439,455,459,489] pokeweed antiviral protein,[373] MAB linked to chemotherapeutic agents,[490] anti-TAA MAB and chemotherapeutic agents unlinked,[491] or the combination of a chemotherapeutic agent and immunotoxin.[492] Only very preliminary clinical evaluation of some of these methods for purifying bone marrow in vitro has been reported, e.g., chemotherapeutic agents,[493,496] antibodies along with complement,[495-497] or immunotoxins.[498] Antibodies and complement tend to be toxic towards progenitor and other non-neoplastic cells in the marrow, cause clumping of cells, and lead to antigenic modulation.[499] Furthermore, many MAB do not fix complement or require very high antibody concentrations for cell killing.[118] Exposure to multiple antibodies has been demonstrated to be more effective in eliminating neoplastic cells than the use of a single anti-TAA antibody.[487,497] In contrast to the short time needed for the killing of cells by complement and antibody, immunotoxins need hours or days to kill target cells.[500]

Of interest are a number of model studies with immunotoxins aimed at eliminating specific cell types from bone marrow ex vivo. The studies of Uckun et al. with conjugates of pokeweed antiviral protein were discussed previously.[373] In an experiment to simulate eradication of malignant cells from human bone marrow, a mixture of bone marrow and 15% BCL_1 leukemic cells was treated in vitro with ricin A chain conjugated by disulfide interchange to an anti-BCL_1 idiotypic Ig and then adoptively transferred to lethally irradiated mice. There was selective but not total eradication of tumor cells with a loss of no more than 10% of normal bone marrow cells.[29] Another example comes from the studies of Muirhead et al.,[439] who conjugated ricin A chain to an affinity-purified polyclonal Ig against human lambda and kappa chains by disulfide interchange. This conjugate was able to eliminate 99% of surface IgM/kappa-bearing Daudi cells mixed with marrow cells in vitro without demonstrable toxicity to hemopoietic cells.

Several studies have also been carried out to eliminate leukemic T-cells from human bone marrow. Casellas et al.[501] produced a ricin A-chain immunotoxin using the method of Jansen et al.[502] with MAB T101, an IgG2a against the T1 65-kdalton glycoprotein differentiation antigen expressed by T-cells and CLL cells but not by normal myeloid and erythroid progenitor cells. Assay of the conjugate in a cell-free protein synthesis system showed that 1.5 to 2 mol of active A chain was present per mole of IgG2a. Fluorescence assay showed that all antibody activity was retained. In a clonogenic assay, CEM cells treated with the specific conjugate at 10 nM in the absence of ammonium chloride exhibited 14% survival at 24 hr. At this conjugate concentration,

substantially all target antigen sites were occupied when cell numbers did not exceed 2×10^7 mℓ. Almost all CEM cells (99.99%) treated with the specific conjugate plus 10 mM ammonium chloride were killed at 8 hr. (The more rapid killing produced by the ammonium chloride should improve clinical efficacy.) Cells which survived the treatment with conjugate plus ammonium chloride lacked antigen. One million cells from an antigen-positive subclone were all killed by the specific toxin under identical experimental conditions, indicative of a 6-log or better efficacy factor. This treatment allowed survival of hematopoietic progenitor cells. Cytotoxicity was blocked by unconjugated antibody. A conjugate prepared with anti-CEA antibody was without cytotoxic effect. The T1-negative cell lines, Daudi and Raji, were not inhibited by the T101 conjugate up to 10 nM, although their sensitivity to ricin or ricin A chain is similar to that of CEM cells. An advantage in clinical application pointed out for A chain conjugates as opposed to whole ricin conjugates was the fact that the treated marrow would not have to be carefully washed to eliminate residual whole toxin before re-infusion into a patient.

An in vitro T-cell leukemic model was used to investigate conjugates of whole ricin toxin prepared by disulfide interchange.[378] The antibodies were T101, which gave the most effective conjugate, and 3A1, an IgG1 which recognizes a 4-kdalton antigen on T and some early myeloid cells. Residual free ricin was removed from the product by protein A-Sepharose chromatography and free antibody by Sepharose 4B chromatography. Retention of toxin activity in the conjugates was confirmed by a cell-free protein synthesis assay. Either specific conjugate strongly inhibited antigen-bearing CEM and 8402 ALL cell lines by approximately 2 logs at a concentration of 0.1 nM but not a B-cell leukemia (8392) or promyelocytic leukemia (HL60) cell line, although colony formation by bone marrow progenitor cells was inhibited approximately 30% at this concentration. The cytotoxic effect of the T101 conjugate toward CEM cells was maintained in mixtures of CEM and bone marrow progenitor cells. Release of free ricin from treated cells did not occur in sufficient amounts to present a clinical hazard. The results of this study, as well as that of Stong et al.,[488] showed that the effectiveness of whole ricin-antibody conjugates was not solely determined by the amount bound to the cell surface, probably because of problems with internalization.[378]

Immunotoxins constructed with ricin A chain were more potent when two or more conjugates directed against independent epitopes were combined.[503,504] In a study using immunotoxins constructed with intact ricin and four MAB against independent epitopes on leukemic T-cells from acute T-cell leukemia patients (i.e., MAB T101, G 3.7, 35.1, and TA-1), the immediate toxicity (protein synthesis inhibition) was compared with the inhibition of clonogenicity by these conjugates. Inhibition of protein synthesis in the presence of lactose in vitro correlated well with the extent of antigenic expression on the surface of the leukemia cells. The conjugate constructed with T101 gave the fastest rate of protein inhibition. A cocktail of four cell conjugates exhibited kinetics that were as fast or faster than the kinetics of the T101 conjugate. On colony inhibition assay in the presence of lactose, all four immunotoxins were specifically cytotoxic and a cocktail of the four was more potent than the T101 conjugate alone.[500] In a phase-I trial, a ricin A chain T101 conjugate was used to purge autologous bone marrow of malignant T-cells. In every patient, hematopoetic recovery occurred within normal time periods, but two patients developed severe infection. There was a suggestion of increased fragility of the incubated progenitor cells to freezing.[498,505]

Another approach to potentiating the action of immunotoxins in vitro is addition of ammonium chloride and/or mafusamid or chloroquine during incubation.[373,492,503,505] Douay et al.[505] demonstrated that an MAB T101-ricin A chain conjugate in the presence of ammonium chloride could eliminate 99.9% of target cells without interfering with the proliferative capacity of hematopoietic stem cells. Ricin (10^{-7} M) linked to a

MAB and incubated in vitro in the presence of ammonium chloride could eliminate at least 4 logs of clonogenic tumor cells from a 20-fold excess of bone marrow.[503] This is comparable to the tumor cell kill achieved with 4-hydroxyperoxycyclophosphamide (see below) or with multiple MAB in the presence of complement. Uckun and coworkers[453] have reported that mafusamid along with a ricin-anti P67 MAB conjugate specific for both normal and leukemic T-cells[453] or with a ricin-PAN-B MAB conjugate[492] could eliminate on the order of 7 logs of target cells with minimal toxicity to normal bone marrow progenitor cells. These studies are important because to be useful for the removal of neoplastic cells from bone marrow the carrier MAB should not react with hematopoietic stem cells.

The efficiency of anti-TAA antibodies and their conjugates in eliminating tumor cells from bone marrow will depend upon the variability of expression of the target TAA, the heterogeneity of TAA, and the specificity of the antibody, i.e., the extent of its reactivity with hematopoetic progenitor cells. Several MAB have been compared as regards their efficiency in removing malignant B cells from bone marrow.[503] It is interesting that the efficiency of several anti-B-cell MAB for complement-mediated lysis did not correlate with their effectiveness as a component of immunotoxins.

It has been claimed that HLA-DQ determinants are expressed on mature malignant lymphoma cells but not on normal bone marrow progenitor cells.[506] A panel of polyclonal and monoclonal anti-HLA-DQ antibodies in the presence of complement has been reported to be able to eliminate 98% of clonogenic tumor cells in vitro. Immunotoxins based on these antibodies have not yet been evaluated.

B. Purging of Immunocompetent Cells from Bone Marrow to Prevent Graft-Vs.-Host Disease (GVHD)

Another use of antibody-linked agents in the management of human leukemias is the prevention of GVHD, which develops in approximately 50% of patients given bone marrow grafts from HLA-matched siblings.[482] Attempts are being made to control GVHD by in vitro depletion of T-cells from grafts prior to transplantation by immunophysical methods,[507] by administration of a cocktail of anti-T-cell MAB,[482,508] or immunotoxins.[509-511] In a pilot clinical study, 14 patients were given bone marrow treated in vitro with an immunotoxin constructed with intact ricin bound to three different, well-characterized anti-T-cell MAB. In vitro analysis of treated marrow samples revealed significant T-cell depletion but sparing of hematopoetic cells. No toxicity could be seen in the patients transfused with the treated bone marrow. According to these investigators,[510] there was prompt acceptance of the grafted bone marrow in their patients. In another study based on eight patients given autologous bone marrow, there was considerable reduction of the risk of developing GVHD when mature T-cells were removed from the bone marrow graft with anti-T12 MAB and complement.[497] However, there is evidence from other studies that the acceptance of bone marrow grafts is inhibited after T-cell depletion.[512] In regard to patients with developed GVHD, 15 patients with steroid-resistant severe GVHD were given various doses of one or more anti-T-cell MAB (a total of four MAB against independent epitopes was used). Most of the patients treated with MAB needed platelet infusions. However, the amount of platelets needed was not different from that needed by patients subjected to other therapeutic regimens. Six of ten patients given intermediate doses of the MAB had evidence of at least partial improvement. None of the patients developed detectable evidence of immunization with mouse Ig. Apart from fever and chills, there was no evidence of toxicity.[513]

C. Preliminary Experience with Drug Antibody Conjugates in Patients

1. Melanoma

In a study by Ghose et al.,[276] 13 consecutive patients with inoperable recurrent malignant melanoma were treated with chlorambucil bound to goat or rabbit antihuman melanoma Ig. The next consecutive 11 patients fulfilling the criteria for admission into this study were treated with chemotherapy only, i.e., dimethyltriazenoimidazole carboxamide (DTIC). Follow-up was for a minimum of 29 months or until death. Two patients showing an objective response to immunochemotherapy had disease confined to lymph nodes and cutaneous sites; five others showed stabilization of cutaneous, nodal, and visceral metastases; and six patients showed progression of their disease. The median survival of the responders and stabilizers was 20 months, but only 3.5 months for patients with disease progression. None of the 11 patients treated with DTIC had objective tumor regression and all died within 11 months of the start of treatment, with a median survival of 3 months. Immunochemotherapy significantly prolonged survival compared to that in the DTIC-treated group ($p < 0.05$). No hematological or renal toxicity was detected after immunochemotherapy.

Recently, 3 of 12 melanoma patients given an IgG_3 mouse MAB against the GD_3 antigen showed "major" tumor regression. Patients given more than 80 mg/m^2 of the antibody had an inflammatory reaction at tumor sites. Examination of biopsied tumor tissue revealed complement deposition, infiltration of mast cells and lymphocytes, and mast cell degranulation.[497] A phase-I trial of an immunotoxin constructed with ricin A chain has also been completed. It has been reported that there was no major problem with toxicity, contraindicating the clinical use of the immunotoxins. Three main toxicities observed in this trial were lethargy and malaise, evening fever, and reversible hypoalbuminemia without proteinuria.[514]

2. Neuroblastoma

Melino and co-workers have treated a number of neuroblastoma patients with antibody-conjugated drugs.[515-519] The carriers were allogenic polyclonal antineuroblastoma antibodies prepared by immunizing haploidentical volunteers with irradiated neuroblastoma cells and fractionation of plasma with cold ethanol.[517] One antibody reacted with seven out of eight neuroblastoma cell lines tested.[516] Preparations could be obtained that did not contain anti-HLA antibodies or cross-react with normal human brain.[516] Conjugation of either daunorubicin or chlorambucil was carried out using a water-soluble carbodiimide. A total of 3 to 4 mol of drug was incorporated per mole of Ig. SDS-PAGE showed polymers, but both daunorubicin and chlorambucil conjugates were reported to retain essentially full antineuroblastoma activity as determined by membrane immunofluorescence.[517] In a cytotoxicity assay using human neuroblastoma cells exposed in vitro to a conjugated daunorubicin concentration of 20 μg/mℓ, the conjugate produced a 60% kill compared with 35% for a mixture of free drug and antibody.[515] Two patients treated with daunorubicin conjugates showed good initial responses, then relapsed and died at 3 and 8 months. Four remained alive and disease-free after 6 to 19 months of treatment. No drug side effects were observed.[515] They later reported that 9 of 12 patients treated with conjugates of both daunorubicin and chlorambucil (30 mg IgG/kg, twice a week) showed marked "antitumor responses" with no detectable anti-idiotypic or antiallotypic antibodies or other blocking factors.[516] No toxic side effects were noted. A recent report from this group[517] dealt with seven patients with advanced neuroblastoma treated following a protocol consisting of one conjugated chlorambucil (0.5 mg/kg) and two conjugated daunorubicin (1 mg/kg) injections per week for 1 year. Lower catecholamine levels were noted in 7 out of 7 patients. Partial regression of tumors occurred in patients with stage IV disease, while those with less than stage IV cancer had no evidence of disease after 3 years.

In a single patient with metastatic disease who had prior chemotherapy and surgery, treatment with conjugated chlorambucil alone and then with conjugated chlorambucil and daunorubicin led to initial regression of lesions followed by resumption of growth. The lack of therapeutic response was attributed to drug resistance since the antibody bound to tumor from the recurrent primary removed at operation.[519]

VII. MODE OF ACTION

Although knowledge of the mode of action of anti-TAA antibody conjugated agents should contribute to the design of more effective conjugates, this area of investigation is only now accelerating; the focus hitherto has been on documenting cytotoxicity in vitro and in vivo. In investigating the mode of action, it is necessary to consider: (1) the antitumor effect of an antibody alone; (2) additive or synergistic effects of antibody and cytotoxic agent unlinked; and (3) alterations in the activity of an agent and in its pharmacokinetics as a result of linkage to antibody.

A. Effect of Antibodies Alone on Tumor Growth In Vivo

The experience of Ghose et al.[520,521] and that of others[491,515-524] shows that only a very small tumor load of 10^5 to 10^6 cells can be eradicated by passive serotherapy with polyclonal anti-TAA antibody. Limitations of conventional antitumor sera include: (1) difficulty in raising amounts adequate for therapy; (2) relatively low titer due to repeated absorptions with normal tissues for obtaining tumor specificity; and (3) persistence of nonspecific Ig. However, increasing numbers of reports are appearing on MAB serotherapy of experimental and human tumors, especially leukemia and lymphoma, as summarized by Ritz and Schlossman.[118] Also, a number of phase I feasibility studies with MAB in human cancer have been listed by Dillman and Royston.[525] Ideally, antitumor MAB should react with the target tumor only, but so far, MAB that react with tumor as well as some normal cells (and are not significantly toxic to the recipient) have been the ones most used in human serotherapy. Treatment in humans involved different types of leukemias and lymphomas with MAB of different specificities[118] and melanomas with MAB against P97, GD_3, and a proteoglycan antigen.[526,527] With respect to B-cell CLL, Dillman et al.[314] treated two CLL patients with MAB T101 directed against a 65-kdalton protein expressed by normal and malignant T- and B-cells. Administration of the antibody resulted in rapid clearance of circulating leukemia cells, but they soon returned to pretreatment levels in both patients. Both had adverse reactions to the MAB infusion. On the other hand, Miller et al.,[312] using a monoclonal anti-idiotypic antibody, induced a dramatic response without any acute or chronic toxicity in a patient with B-cell lymphoma. "Major tumor regression" occurred in 3 of 12 melanoma patients treated with MAB R_{24} against GD_3[527] but in none of the 5 patients treated with the other two MAB.[526] However, generally the results of clinical trials of anti-TAA antibodies alone in cancer patients have been disappointing.[118] It should be realized that antibodies themselves are not inherently cytotoxic and their binding to a target cell membrane does not affect growth except in rare instances.[12] Possible mechanisms by which they could produce tumor inhibition in vivo include complement (C)-mediated cytotoxicity, clearance by reticuloendothelial cells, and antibody-dependent cell-mediated cytotoxicity.[525] Investigations into the mechanism of tumor inhibition by MAB have revealed that those of the IgG class inhibit tumor cells by C-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity, whereas MAB of the IgM class inhibit tumors only by C-dependent cytotoxicity. IgM anti-TAA MAB have failed to show any effect on tumor growth in vivo.[118,301] This suggests that C-dependent cytotoxic mechanisms do not contribute in a major way to tumor inhibition. This is consistent with our observations using conventional xenogenic sera.[520] In leukemia pa-

tients, administration of MAB is followed by a rapid fall in circulating leukemic cells due to their sequestration in the reticuloendothelial system. However, this reduction is only transient.

In summary, the results of serotherapy with anti-TAA MAB in experimental and human cancer show transient tumor suppression, a modest increase in survival, and eradication of an individual tumor burden of approximately 10^5 cells. The magnitude of the clinical problem can be appreciated by the fact that the tumor burden is very high in patients refractory to standard therapy (e.g., 10^{12} cells). In the patient described by Miller, the infusion of 1 mg of MAB removed approximately 2×10^{11} tumor cells, yet the influence on progression of the disease was negligible, i.e., the total tumor cell population recovered in 24 hr.[312] It is, therefore, essential to increase the cytodestructive effect of anti-TAA antibodies, e.g., by their linkage to cytotoxic agents.

B. Additive or Synergistic Effects of Cytotoxic Agents and Antibodies Unlinked

Synergistic tumor inhibitory effects of anti-TAA antibodies plus cancer chemotherapeutic agents have been demonstrated both in vitro[528,529] and in vivo.[530] Ghose et al. confirmed synergism with chlorambucil,[395,398] MTX,[345] and Trenimon[292] in tumor-inoculated mice. However, the full extent of tumor inhibition by cytotoxic agent-anti-TAA antibody conjugates cannot be explained entirely by synergism,[395,398] except that with the alkylating agent Trenimon there was no difference in the extent of tumor inhibition by Trenimon linked to anti-TAA antibody and equivalent amounts of the drug linked to a nonspecific IgG together with the anti-TAA antibody unlinked.[292]

Exposure of tumor cells to certain cancer chemotherapeutic agents or metabolic inhibitors has been shown to inhibit the synthesis of complex cell surface lipids and render them more susceptible to antibody and complement.[531] If the toxic moiety in conjugates is surface active, then the agent and antibody may act additively or synergistically. Damage to the cell membrane by the conjugate or its components may also facilitate entry into cells and allow access to intracellular targets. Potentiation of the antitumor effect of ionizing radiations by anti-TAA antibodies has been observed both in vitro[532] and in vivo.[469] Ionizing radiations may also interfere with the permeability of plasma membranes.[533]

C. Alterations in the Action of an Agent and in Its Pharmacokinetics as a Result of Linkage to Antibody

The therapeutic index is the critical factor in the application of antibody-conjugated agents. There may well be a decrease in drug activity in a conjugate compared to the free agent, but the systemic toxicity of that conjugate may be lower than that of the free drug, allowing a compensating higher dose of conjugated drug. Gallego et al.[334] have emphasized that tumor-selective cytotoxicity in vivo is a more important parameter than cytotoxicity in vitro.

One approach to an insight into the mode of action of antibody-linked cytotoxic agents is to study and compare the effect of intact conjugates and their components (especially the cytotoxic entity): (1) at the molecular level by investigating interaction with putative target molecules of the agent in cell-free system, (2) at the cellular level by investigating effects of cells in culture, and (3) at the level of intact animals by investigating effects on tumor-bearing animals or cancer patients. In evaluating the mechanism of action on tumor cells in culture, factors that need consideration include: (1) access of agents, free and conjugated, to target molecules, especially if these are intracellular; (2) the catabolism of conjugates and their components during transit to target molecules; and (3) the removal (e.g., efflux or otherwise) of pharmacologically active moieties from the target compartment. In intact animals, additional considerations include: (1) stability, especially the retention of agent and antibody activities in

conjugates while in the circulation; (2) factors that govern homing and catabolism during transit from the vascular to the extravascular compartment in solid tumors; and (3) alterations in the pharmacokinetics and systemic toxicity of agents as a result of conjugation. Although somewhat sketchy, information at these three levels is gradually emerging. In particular, conjugates containing MTX, adriamycin/daunorubicin, or chlorambucil and immunotoxins constructed with the A chain of ricin have been investigated.

1. Action at the Molecular Level

Linkage of cytotoxic agents to Ig or other macromolecular carriers may either augment or inhibit the effect of the agent on its target molecules. For example, linkage of chlorambucil to Ig inhibits its catabolism or transformation to inactive forms[297] and thus preserves alkylating activity, which is accepted as the basis of its cytotoxic action. On the other hand, there may be loss of activity of the agent during or after conjugation due to various causes that include inappropriate reaction conditions leading to chemical damage of the agent, e.g., loss of activity of MTX during conjugation following the "mixed anhydride" method[345] and steric hindrance. Ghose et al. observed that adriamycin conjugated to IgG or high molecular weight dextran does not bind to DNA in vitro.[13] Intercalation into DNA is thought to be essential for the expression of the cytotoxic effect of these agents.[12] The elegant studies of Trouet et al.,[339] Monsigny et al.,[338] and Shen and Ryser[333] have demonstrated that intralysosomal release of daunorubicin can restore the cytotoxic action of inert conjugates. Ghose et al. also demonstrated that MTX conjugated to IgG or its $F(ab')_2$ moiety is only half as potent in inhibiting its target enzyme DHFR as the free drug.[299,345] In regard to immunotoxins, it has been demonstrated that intact diphtheria toxin or ricin inhibits protein synthesis in cell-free systems at least 1000-fold less effectively than the free A chains. Thus lysosomal cleavage of the A chain appears to be essential even though the mode of transport of the cleaved A chain to the cytosol is not well understood.[534,535] Surprisingly, conjugates in which the A chain of ricin was linked to the F(ab) moiety of IgG were as effective inhibitors of protein synthesis in cell-free systems as the free A chain, irrespective of whether the linkage between the A chain and F(ab) was cleavable.[536] The difference in the behavior of B chain-linked and F(ab)-linked A chains of ricin in cell-free systems has been attributed to the inflexibility of the covalent and noncovalent bonds between A and B chains. It should be stated that antibody or other macromolecule-linked agents may exert their cytotoxic effect via mechanisms that are not usual or predominant with the free agent. Thus intracellular DNA is regarded as the principal target of alkylating agents like Trenimon and chlorambucil or intercalating agents like adriamycin and daunorubicin. However, all these agents are also surface active and conjugates containing them could damage cell membranes and be cytotoxic even in the absence of endocytosis.[292,537]

2. Action on Target Cells In Vitro

A large number of antibody conjugates have been produced and the specificity of their binding to target cells in vitro has been demonstrated.[14] However, on comparing the biological effectiveness of antibody-linked agents with that of the agent, free or linked to nonspecific Ig, two patterns of inhibition of target cells in vitro have been observed. In some experiments, the order of tumor inhibition has been free drug > drug linked to anti-TAA antibody > drug linked to nonspecific Ig. This pattern has been reported with Trenimon,[292,538] MTX,[345] purothionin,[409] adriamycin,[13,264] and vindesine.[431] The other pattern of inhibition is antibody-linked agents > free drug > or = drug linked to nonspecific Ig, and has been observed with other drugs such as

chlorambucil[394] and with drugs in the first group but assayed by different methods on different cell lines, e.g., daunorubicin[415,416,539] and vindesine.[430] This observed difference in the relative effectiveness of conjugates may be due to differences in the carrier antibody and its target antigen, the nature of the agent, especially its site of action and its transport characteristics, the method of linkage, the cell line used, and the method used for assaying tumor inhibition. For example, Embleton et al.[431] have observed that when the target cells were exposed to vindesine and its conjugates for 24 hr free drug was 2000 times more cytotoxic than the antibody conjugate; with shorter exposure the free drug was far less effective. For agents that need to be internalized to exert cytotoxic effects, it is essential that the carrier antibody is capable of being endocytosed after binding to its receptor on the cell surface. Endocytosis can follow capping of the cell surface-bound antibody conjugate,[297] although this may not be always so.[292]

For the construction of tumor-specific immunotoxins, it is essential either to cleave off or inhibit indiscriminate binding via the B chain to various nonmalignant cells.[340] Since the target molecules of most of the protein toxins are intracellular,[14,340] binding of immunotoxins to the surface of target cells with subsequent endocytosis is a prerequisite for their cytotoxic action. It is, therefore, not surprising that most of the immunotoxins without B chains show exquisite specificity of binding to tumor cells in vitro and the cytotoxic potency that is higher by several magnitudes than that of the free A chain or A chain linked to a nonspecific carrier. The latter two are endocytosed very poorly by most tumor lines in vitro.[540] It should be stressed that irrespective of whether the activity of antibody conjugates exceeds that of the free drug, all agents have been rendered tumor specific after linkage to anti-TAA antibodies, i.e., antibody-linked agents have been shown to be more inhibitory towards target cells than towards nontarget cells and more potent toward target cells than equivalent amounts of the agent linked to nonspecific Ig.[14,340]

The sequence of events following the binding of antibody conjugates to the surface of target cells in vitro and the path of uptake of antibody-linked chemotherapeutic agents have been elucidated by a number of elegant studies. Using appropriately labeled daunorubicin and its conjugates, Arnon has observed that the order of rate of uptake and amount of drug taken up by cells and nuclei was free drug > drug bound directly to the antibody > drug bound to antibody via a dextran bridge > drug bound to nonspecific Ig.[539] As already stated, this drug needs to be cleaved off the carrier in lysosomes to be cytotoxic.[333,339] It is possible that the slow release of the active drug from the inert intracellular conjugate constitutes an "intracellular depot" effect adding to the potency of the conjugated agent.

Studies by Uadia et al.[295,307,408] have contributed to the understanding of the mechanism of action of MTX-antibody conjugates. When mouse EL4 lymphoma cells were incubated in vitro, the rate at which the tumor cells took up MTX conjugated with an anti-TAA antibody was much slower than the rate of uptake of free MTX. However, as early as at 6 hr of incubation, the net uptake of conjugated MTX exceeded that of the free drug (or drug linked to nonspecific Ig). Furthermore, when human melanoma M21 cells were incubated in vitro with MTX, free or conjugated to several different anti-TAA antibodies, the amount of uptake of conjugated MTX corresponded to the amount bound at equilibrium at 0°C and the titer of the antibody. At 6 hr, more MTX was endocytosed when linked to antibodies than when linked to a nonspecific Ig or free. Furthermore, the efflux of conjugated MTX was much slower than that of free MTX, resulting in the maintenance of prolonged high intracellular levels of MTX. It had previously been demonstrated that more chlorambucil was taken up by EL4 cells in vitro when the drug was conjugated to an antibody than when conjugated to a nonspecific IgG.[541] These results show that more cancer chemotherapeutic agents are endocytosed by tumor cells when they are linked to an antibody than to a nonspecific

protein. At least with MTX-antibody conjugates, the amount of drug taken up as the conjugate exceeds the uptake of the free drug. Some tumors such as the EL4 lymphoma cells show high endocytotic activity and can take up and retain higher amounts of MTX linked to nonspecific Ig than the free drug.[295]

To elucidate the uptake of immunotoxins, FitzGerald et al.[461] designed a study using KB cells and *Pseudomonas* exotoxin conjugated to an antitransferrin antibody. They demonstrated binding of the intact conjugate first to the cell surface with "clustering" and then prominent localization in the coated pits. This was followed by the rapid transit of all the components of the conjugate to receptosomes. The uptake of the conjugate was inhibited by free antibody but not by the free toxin, confirming antibody-mediated endocytosis. Using a ricin A chain immunotoxin adsorbed on colloidal gold particles, Carriere et al.[542] observed internalization of conjugates via either coated pits or noncoated microinvaginations. Conjugates reached receptosomes of lymphoid cells within 15 min of incubation at 37°C and 50% of the intracellular immunotoxin reached lysosomes within 30 min. Apart from the ultrastructural studies on the uptake of immunotoxins, insight into their mechanism of action has been obtained from studies of kinetics of cytotoxic action and on the observed potentiation of their action by lysosomotropic amines and carboxylic ionophores such as monensin.[543] In a study on the kinetics of cytotoxicity produced by intact ricin and immunotoxins consisting of the A chain linked to antibodies against several cell surface antigens, it has been observed that there is usually a lag between exposure of cells to immunotoxins and the beginning of inhibition of protein synthesis. This suggests that immunotoxins must be processed after endocytosis to gain access to target enzymes in the cytosol.[543] It has already been stated that in cell-free systems, the activity of the A chain of protein toxins is lowered when it is linked to the B chain. Lysosomal processing and liberation of A chains from the toxin molecule is thus essential for the full expression of the cytotoxic potential of the A chain. This is consistent with the observation of Masuho et al.[536] that ricin A chain linked to F(ab) moieties by mercaptoethanol-susceptible bonds was more cytotoxic in culture than when linked via mercaptoethanol-resistant bonds. The released A chain may have greater access to the cytosol compartment. As already stated, the A chain linked to an F(ab) moiety was as effective in inhibiting protein synthesis in cell-free systems as the free A chain. This indicates that release of the A chain from the immunotoxin is not important at the ribosomal level, but the A chain must be directed to the ribosome after binding of the conjugates to the cell surface. In most in vitro studies, the rate of inhibition of protein synthesis in exposed cells was much slower with immunotoxins than with intact ricin. This would indicate that the B chain favors special receptors allowing rapid entry or that the B chain facilitates the transport of the A chain into the cytosol compartment. The demonstration by McIntosh et al.[467] that the addition of free B chains potentiates the action of cell-bound antibody-ricin A chain conjugates (which by themselves were not effective), even when the B chain itself does not bind to the cell surface, suggests that a weak noncovalent linkage of the B chain to the cell-bound conjugate facilitates the internalization of the complex. The studies on the kinetics of action of immunotoxins further revealed that the rate of inhibition of the intracellular target enzymes depended upon the number of toxin molecules bound to the surface. The nature of the carrier moiety of the A chain that binds to the cell surface and of the receptors (i.e., antigen) on the cell surface is also important in determining the potency of conjugates. For example, immunotoxins constructed with IgG antibodies were more effective than those constructed with IgM antibodies of identical specificity.[543] Furthermore, A chains linked to $F(ab')_2$ moieties are more cytotoxic than those linked to F(ab).[536] This may be due to greater ease of capping or other modes of internalization of the bound A chain. In none of these studies was there evidence that any significant amount of toxin[542] or drug

moiety[295,539] was cleaved off the cell-bound conjugate and then transported inside the cell using antibody-independent transport mechanisms.

The potentiation of the action of immunotoxins by lysosomotropic amines and monensin has also been of help in elucidating the mechanism of action. Their effectiveness may be based on either alteration of traffic between intracellular compartments allowing the A chain greater access to the cytosol or inhibition of degradation of the A chain in the lysosomal compartment.[542,543] The role of lysosomal processing in potentiating the action of conjugates appears to vary from one conjugate to another. It is possible that fully potent daunorubicin is released from relatively inert conjugates by lysosomal digestion. However, in the case of MTX, Ghose et al. failed to detect any catabolic fragment that is more active than the parent MTX-IgG conjugate.[295] With immunotoxins, even after the release of A chain, it appears that there is the need for appropriate control of the intracellular traffic so that the free A chain gains access to the cytosol compartment.[543] Prevention of excessive and inappropriate catabolism in the lysosomal compartment is obviously also important.

3. Action In Vivo

Tumor-selective localization of the carrier anti-TAA antibody is the basis of antibody-targeted drug therapy. Specific accretion of anti-TAA antibodies, especially MAB and their reactive fragments, has now been demonstrated in many tumor-bearing animals and cancer patients (see above). However, there are only a few studies in which antibody-mediated tumor localization of the bound cytotoxic agents has been adequately investigated in vivo. The study of Uadia et al. with ascites EL4 lymphoma-bearing mice confirmed higher tumor uptake of an antibody conjugate in vivo.[307] For example, at 2 hr after administration, the uptake of MTX given as the antibody conjugate was 2.5 times the uptake of MTX given as a nonspecific Ig conjugate and 6 times the uptake of free MTX. MTX-conjugated to Ig, irrespective of specificity, was retained in all tissues including EL4 cells much longer than free MTX, and MTX conjugated to anti-EL4 IgG was retained longest in the tumor cells. The levels of intracellular MTX after the administration of free or conjugated MTX exceeded the intracellular level of DHFR. Thus, the greater uptake of antibody-conjugated MTX and its prolonged retention by tumor cells in vivo could explain the therapeutic effectiveness of MTX-antibody conjugates in EL4 lymphoma-bearing mice.[345] More interestingly, the amounts of MTX taken up by EL4 cells in vitro correlated well with its uptake in vivo and with the therapeutic effectiveness of the test agents, i.e., antibody conjugate > nonspecific Ig conjugate > free drug. The therapeutic effectiveness of the test agents in melanoma xenograft-bearing nude mice also followed the order of in vitro uptake of MTX, free or conjugated.[302] In a previous study, Ghose et al. demonstrated tumor-selective localization of these antibodies in human melanoma xenograft-bearing nude mice.[139]

These and other studies[540] on the distribution of cytotoxic agent-antibody conjugates in vivo have demonstrated that linkage of agents to macromolecular carriers alters their pharmacokinetics. As already stated, in EL4 lymphoma-bearing mice, Ig-conjugated MTX was cleared slowly and persisted in all tissues including blood, whereas free MTX declined rapidly after reaching peak levels at 1 hr. If the conjugated agent is active, such prolonged elevated levels of the conjugate in normal tissues (and tumor) may account for the increase in nonspecific cytocidal effect in vivo (i.e., increased systemic toxicity) observed with MTX-containing conjugates. Furthermore, prolonged slow release of the agent from the conjugate can also add to its cytotoxicity, especially if the released agent is more potent than the parent conjugate (i.e., the so-called depot effect). Tumor cells that have high rates of endocytosis of serum proteins[544] are likely to be selectively damaged by such nonspecific conjugates. However, linkage of other

agents to Ig has led to a reduction in their systemic toxicity, e.g., Trenimon,[292] adriamycin,[13] and daunorubicin.[539] The reasons for the reduction in the systemic toxicity of conjugated adriamycin or daunorubicin would include the inability of the parent conjugate to bind to DNA and the necessity for lysosomal processing for potentiation. Also, altered physicochemical properties (e.g., hydrophobicity) of the conjugate may prevent access of the conjugated drug to target molecules in myocardial cells, the susceptibility of which limits the dose of these drugs. It is also possible that the rate and extent of activation of conjugated adriamycin/daunorubicin is lower in myocardial cells. The reduction in the systemic toxicity of conjugated Trenimon is not due to the reduction in its alkylating activity because free Trenimon is more potent in vitro than equivalent amounts of Ig-linked active Trenimon, i.e., Trenimon retaining equivalent amounts of alkylating activity which is the basis of its cytotoxic action.[292] Ghose et al.[292] and Linford and Froese[538] have observed that Ig-bound Trenimon is not endocytosed in vitro. This may explain the relative lack of a cytotoxic effect of the conjugate in vitro and its low systemic toxicity. In any case, reduction in the systemic toxicity of these agents in conjugate form allows their administration in higher doses[264,292,539] that can cause tumor inhibition by the stipulated or alternate mechanisms of action (e.g., membrane damage).

In studying the mechanisms of action of antibody conjugates in vivo, one should also consider other factors that determine the ability of the conjugates to home to tumor cells so that they can exert their effect on or inside the target cells. These include stability of the conjugates in circulating blood, their rate of clearance, and retention of antibody activity in the carrier moiety. The factors that determine the clearance of carrier antibodies have already been discussed (see above). The results of a recent study[540] on the pharmacokinetics of an immunotoxin in rabbits are in agreement with those of Uadia et al.[295] in EL4 lymphoma-bearing mice. These studies show that antibody conjugates are fairly stable in circulation and retain both drug and antibody activities and their initial half-life in circulation is fairly long. The factors that determine tumor-selective localization of antibody conjugates will include: (1) the size and charge of the conjugate; (2) its capacity to cross capillary walls; (3) its rate of diffusion and susceptibility to degradation in the tissue space; (4) the presence or otherwise of the Fc moiety of Ig and the B chain of toxins; (5) the specificity of the carrier antibody; (6) the presence in the circulation of TAA, competing antibodies, and immune complexes; and (7) tumor vascularity. These have been discussed previously. Vascular changes in tumors, e.g., large numbers of newly formed vessels, associated increased vascular permeability, and impaired drainage, may also allow intratumoral localization of conjugates in amounts higher than in normal tissues. This may explain the antitumor effect of agents linked to nonspecific Ig and of daunorubicin-antifibrinogen antibody observed by Lee et al.[413,414]

When one considers the alterations in the pharmacokinetic properties of agents when linked to Ig and the factors that determine the transport of active conjugates from the vascular compartment to the milieu of cells in solid tumors, it is not surprising that there are wide discrepancies between the potency of conjugates on cells in vitro and in vivo. For example, with Trenimon, MTX, and adriamycin, the conjugates were consistently less potent than equivalent amounts of free drugs in vitro. In contrast, in tumor-bearing mice, conjugates were by far the most effective. Even nonspecific Ig conjugates, which were usually least effective in vitro, were more potent than the free drug in vivo. The factors that contribute to the effectiveness of nonspecific Ig-bound agents include: (1) protection of drug activity; (2) altered pharmacokinetics, i.e., prolonged serum and tissue half-life; (3) the "depot" effect; and (4) increased endocytosis of conjugates by tumor cells. It is interesting that the potency of conjugates in vivo correlates better with net uptake of the agent in vitro. Arnon,[539] Arnon and Sela,[357]

and Linford and Froese[538] have also noticed the discrepancy between the in vitro and in vivo potency, which is best illustrated by immunotoxins. Although most eradicate tumor cells very selectively and effectively in vitro,[14,340] only in a few studies were they effective in vivo.[29,545,546] Interestingly, these were not "solid" tumors.

VIII. PROSPECTS FOR THE FUTURE

Various agents, from radioisotopes to proteins, have been conjugated to antibodies against widely differing antigens and the conjugates tested for cytotoxicity using experimental systems ranging from simple cell cultures to models in which human tumors have been xenografted in nude mice and occasionally in patients with advanced cancer. Results in vivo have varied from marginally significant inhibition of tumor growth, to prolongation of survival, to cure of a proportion of the treated animals.[14,29,310,469] One may ask: how close are investigators to effective clinical treatment modalities using targeting by antibodies? In this respect, the diagnostic application of antibody localization is substantially ahead of any treatment modality.[547] For many years, the only clinical trial of drug-linked antibodies in the literature was that reported by Ghose et al.[276] The therapeutic application of radiolabeled antibodies in patients was also investigated by only two groups.[470,548] This shows that the concept of antibody-mediated drug targeting is straightforward, but its realization has turned out to be difficult. However, the availability of MAB and the recent success in the use of antibody-linked toxins to eliminate tumor cells from bone marrow have given renewed impetus to investigation. This discussion of the limitations of cancer treatment with antibody-targeted agents and the possible approaches for overcoming some of them should make it clear that in spite of the recent surge of activity in this field there has to be substantial progress in all the components of this approach to cancer therapy before its application to patients. For example, it will be useful to study the TAA profile of various human tumors and document the biological role of the TAA and their behavior in regard to modulation, capping, and endocytosis. An accessible and updated data base on available anti-TAA antibodies and their target TAA will be of help.

The species of origin of a MAB, its class, and subclass may determine effectiveness as a carrier and its immunogenicity and should therefore be documented. It is possible that with time there will be increasing numbers of anti-TAA MAB that have high affinity and adequate tumor specificity. Absolute tumor specificity may neither be achievable nor necessary. It is possible now to isolate the genes that encode for a given MAB and introduce them back into lymphoid or mouse myeloma cells.[549-551] Using plasmids containing Ig cDNA, coexpression of the light and heavy chains of human Ig has been observed in *E. coli*,[552] and the synthesis, processing, and secretion of functional antibodies have been demonstrated with the yeast, *Saccharomyces cervisiae*.[553] In addition to the coding sequences for Ig chains, the mammalian transcription units that have been transfected include genes that allow bacterial selection (e.g., neomycin-resistant gene) and viral or mammalian promoter, splice, and polyadenylation sites.[554] Furthermore, oncogenic DNA viruses (e.g., SV 40 and Epstein Barr virus[555] and defective retroviruses[556,557]) have been used successfully to insert genes into human B-lymphocytes and bone marrow cells. It should be stated that at present the yield of functionally active Ig from these expression systems is very poor for reasons that include the lack of appropriate association between light and heavy chains and adequate post-translational glycosylation and secretion.

DNA recombinant technology has also made possible the construction of chimeric antibody molecules that contain mouse or rat variable regions having a defined specificity and high affinity and human constant regions that could reduce Fc-related antigenicity and confer therapeutically useful functions such as binding to C1Q or Fc re-

ceptors on certain cell types.[558-561] Furthermore, chain switch in Ig can be achieved using a number of methods[562,563] including UV irradiation.[564] This is especially important in the context of MAB of human origin, most of which appear to be of the IgM class.[565] Methods are also becoming available for the production of myeloma transfectants that secrete $F(ab')_2$ or monovalent Ig fragments either completely lacking the Fc moiety[566] or in which the CH_2 and CH_3 domains are substituted by an enzyme moiety.[567] Recombinant DNA technology is thus likely to complement the current hybridoma or B-cell immortalization methodology for the production of anti-TAA MAB for targeting. For example, recombinant technology and methods of site-directed mutagenesis can be used to improve the affinity and other therapeutically desirable qualities of human MAB[563] that have been obtained initially with the use of either lymphocytes that have been antigen-primed in vitro[568] or in vivo (e.g., lymphocytes in tumors or in draining lymph nodes). Genetic engineering is also likely to provide in the not far distant future tailored antibody molecules with appropriate polypeptide chains for optimal conjugation to a given protein toxin or a chemotherapeutic agent. This will include hybrid antibody molecules (already constructed by chemical methods[370]) that have one arm of the IgG directed against the TAA and the other against the protein toxin. It may also be possible to obtain homogeneous populations of molecules that contain the functionally active antigen-binding site of the Ig optimally linked to the toxophore group of a protein toxin or biological products with antitumor activity, e.g., tumor necrosis factor, the gene for which has already been cloned and expression observed in *E. coli*.[569] In contrast to conventionally produced MAB that are products of malignant cells, products of transfectomas are more likely to be free of contaminating oncogenic viruses and nucleic acids.

A number of approaches are possible for production of more effective conjugates and for amplification of their antitumor effects. For example, one can select active analogs of chemotherapeutic agents that have a high influx K_M, e.g., the gamma asparatate derivative of MTX which has a Km for influx into L1210 cells exceeding 300 μM compared to 3.3 μM for MTX.[570] This will minimize uptake of the agent by nontarget cells, as in the case of immunotoxins that contain only A chains. The choice of cytotoxic agent will also depend upon the nature of the TAA, the antibody, the susceptibility of the tumor cells, tumor vascularity, and tumor cell heterogeneity. For anti-TAA antibodies and their fragments such as F(ab) that are not endocytosed, surface active agents and radionuclides should be the agent of choice. For tumors that are relatively avascular or contain antigen-negative tumor cell populations, radionuclides are likely to be more effective than other agents since their tumoricidal effect can extend to neighboring cells.

The choice of the method of conjugation is based presently on the preservation of agent and antibody activities. Better insight into the mechanism of action of conjugated agents, their pharmacokinetics, and their catabolism should allow selection of bonds that are optimal for the effectiveness of the conjugate. Methods need to be devised to prevent the premature dissociation of the carrier antibody and the toxophore and facilitate direct transit of the toxophore to its target molecules in vivo. Regiospecific and pH-sensitive linkages are becoming available and may be of help.[571]

Although the use of a "cocktail" of antibodies has not been very impressive in increasing tumor localization of antibodies,[572] there is evidence that mixtures of ricin-containing immunotoxins against three different T-cell antigens were more effective in inhibiting T-cells in vitro than any single immunotoxin.[509] Furthermore, there may be an increase in the selectivity and the extent of damage with the use of bacterial protein toxins that act cooperatively on mammalian cell membranes.[573] Another approach for amplifying the effect of antibody conjugates is illustrated by the synergistic action of

Pseudomonas exotoxin antibody conjugates and adenovirus[460,574] or ricin-containing immunotoxins against neoplastic T-cells and in vitro active congeners of cyclophosphamide[575] and other cancer chemotherapeutic agents.[575-578] The use of lysosomotropic agents and ionophores that potentiate the effects of conjugates has been discussed.[373,453,460,462,578] Furthermore, the use of biological response modifiers may induce the expression of TAA[173] and vasodilators may improve tumor perfusion.[29]

Another approach will be combination immunochemotherapy. For many cancers, multiple agents are more effective than a single agent. One may render antibody-linked agents more effective by combining more than one free or conjugated drug (including radionuclides). To illustrate, let drugs be designated by A or B and antibodies by X or Y, such that conjugates are A-X, B-Y, A-X-B, etc. One approach is to increase the intracellular concentration of a given drug by exposure to both free and antibody-linked agents so that the drug is internalized by receptors for the antibody and for the drug, e.g., A plus A-X. A variation would be to use A plus B-X, which would show whether the best inhibition is obtained by an increased concentration of a given drug or by two different drugs having different modes of action, e.g., chlorambucil and MTX. Another approach would use two or more conjugates made with one agent by linking to antibody directed against different epitopes that cap and endocytose independently, e.g., A-X plus A-Y. A third approach is a form of combination therapy directed by antibodies. One can link two or more different drugs to a given antibody molecule, e.g., A-X-B, or use a mixture of two conjugates in which different drugs have been linked to separate batches of the same antibody, e.g., A-X plus B-X. Limitations arising from the availability of binding sites for X may be overcome if A and B are linked to antibodies against different epitopes, e.g., A-X plus B-Y.

The effectiveness of antibody-linked agents can be further increased by combination with one or more additional modalities of treatment. It has already been pointed out that antibody conjugates are likely to be most effective against tumor microemboli or cells in circulation and therefore it is essential to reduce the tumor burden by surgery and/or radiation. Furthermore, it is possible that exposure to hyperthermia and/or ionizing radiations (either from an external source or appropriate radionuclides linked to the carrier antibody) may have a complementary or synergistic effect. In the authors' laboratory, it has been observed that exposure to MTX and hyperthermia have additive inhibitory effects on several human tumor cell lines in vitro. Interestingly, MTX arrests cells in the S phase in which they are susceptible to hyperthermia. Thus, local or whole body hyperthermia may be a useful adjunct to immunochemotherapy. Further potentiation of the combined effect of immunochemotherapy and hyperthermia may be possible by linkage to the carrier antibody of appropriate metals that, during magnetic induction heating, have much higher energy absorption than body tissues as well as high thermal conduction properties.[579]

The problems, although formidable, are not insurmountable, especially if this modality of treatment is used for the eradication of those tumor cells in circulation or microemboli that escape and therefore limit the effectiveness of other methods of cancer treatment.

REFERENCES

1. Holland, J. F., Karnofsky memorial lecture: breaking the cure barrier, *J. Clin. Oncol.*, 1, 74, 1983.
2. Baserga, R., The cell cycle, *N. Engl. J. Med.*, 304, 453, 1981.
3. Gregoriadis, G., Targeting of drugs: implications in medicine, *Lancet*, 2, 241, 1981.

4. Pozansky, M. J. and Juliano, R. L., Biological approaches to the controlled delivery of drugs: a critical review, *Pharmacol. Rev.*, 36, 277, 1984.
5. Chang, T.-M., Dayord, A., and Neville, D. M., Jr., Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulfide conjugate. II. Biochemical and biologic properties of diphtheria toxin fragment A-S-S-human placental lactogen, *J. Biol. Chem.*, 252, 1515, 1977.
6. Varga, J. M., Hormone-drug conjugates, *Methods Enzymol.*, 112, 259, 1985.
7. Cawley, D. B., Herschman, H. R., Gilliland, D. G., and Collier, R. J., Epidermal growth factor-toxin A-chain conjugates: EGF-ricin A is a potent toxin while EGF-diphtheria fragment A is non-toxic, *Cell*, 22, 563, 1980.
8. Pirker, R., Fitzgerald, D. J. P., Hamilton, T. C., Ozols, R. F., Willingham, M. C., and Pastan, I., Anti-transferrin receptor antibody linked to pseudomonas exotoxin as a model immunotoxin in human ovarian carcinoma cell lines, *Cancer Res.*, 45, 751, 1985.
9. Firestone, R. A., Pisano, J. M., Falck, J. R., McPhaul, M. M., and Kreiger, M., Selective delivery of cytotoxic compounds to cells by the LDL pathway, *J. Med. Chem.*, 27, 1037, 1984.
10. Uchida, T., Yamaizumi, M., Mekada, E., Okada, Y., Tsuda, M., Kurokawa, T., and Sugino, Y., Reconstitution of hybrid toxin from fragment A of diphtheria toxin and a subunit of Wisteria Fluoribunda lectin, *J. Biol. Chem.*, 253, 6307, 1978.
11. Gilliland, G. D., Collier, R. J., Moehring, J. M., and Moehring, T. J., Chimeric toxins: toxic, disulfide-linked conjugate of concanavalin A with fragment A from diphtheria toxin, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 5319, 1978.
12. Ghose, T. and Blair, A. H., Antibody-linked cytotoxic agents in the treatment of cancer: current status and future prospects, *JNCI*, 61, 657, 1978.
13. Ghose, T., Blair, A. H., Vaughan, K., and Kulkarni, P., Antibody-directed drug targeting in cancer, in *Targeted Drugs*, Goldberg, E. P., Ed., John Wiley & Sons, New York, 1983, 1.
14. Ghose, T., Blair, A. H., and Kulkarni, P., Preparation of antibody-linked cytotoxic agents, *Methods Enzymol.*, 93, 280, 1983.
15. Ghose, T., Blair, A. H., Kulkarni, P., Vaughan, K., Norvell, S. T., and Belitsky, P., Targeting of radionuclides and drugs for the diagnosis and treatment of cancer, in *Targeting of Drugs*, Gregoriadis, G., Ed., Plenum Press, London, 1982, 55.
16. Ghose, T., Blair, A. H., Martin, R. H., Norvell, S. T., Ramakrishnan, S., Belitsky, P., and Ferrone, S., Tumor imaging by antitumor antibody linked radionuclides, in *Tumor Imaging*, Burchiel, S., Ed., Masson Publishers, New York, 1982, 167.
17. Ghose, T., Kulkarni, N., Ferrone, S., Giacomini, P., Norvell, S. T., Kulkarni, P., and Blair, A. H., Imaging human tumors in nude mice, in *Radioimmunoimaging and Radioimmunotherapy*, Burchiel, S. and Rhodes, B. A., Eds., Elsevier, Amsterdam, 1983, 255.
18. Blair, A. H. and Ghose, T., Linkage of cytotoxic agents to immunoglobulines, *J. Immunol. Methods*, 59, 129, 1983.
19. Haynie, T. P., III, Hutchins, R. D., and McKelvey, E. M., Selected abstracts on radiolabeled antibodies in cancer, Oncology Overview, 1984, U.S. Department of Health and Human Services, Natl. Technical Information Service, Springfield, Va.
20. Weiss, D. W., Reflections on tumor origin immunogenetically and immunotherapy, *Cancer Immunol. Immunother.*, 18, 1, 1984.
21. Hewitt, H. B., A critical examination of the foundations of immunotherapy for cancer, *Clin. Radiol.*, 30, 361, 1979.
22. Parmiani, G. and Pierotti, M. A., Generation of TSTA diversity: looking for testable hypothesis, *Cancer Immunol. Immunother.*, 14, 133, 1983.
23. Woodruff, M. F. A., Ansell, J. D., Hodson, B. A., and Micklem, H. S., Specificity of tumours associated transplantation antigen (TATA) of clones from the same tumours, *Br. J. Cancer*, 99, 5, 1984.
24. Rogers, M. J., Tumor-associated transplantation antigens of chemically-induced tumors new complexities, *Immunol. Today*, 5, 167, 1984.
25. Harnden, D. G., Genetic change in the cancer cell, *Br. J. Cancer*, 49, 1, 1984.
26. Reddy, E. P., Reynolds, R. K., Santos, E., and Barbacid, M., A point mutation is responsible for the acquisition of transforming properties of the T24 human bladder carcinoma oncogene, *Nature (London)*, 300, 149, 1982.
27. Salmon, D. J., deKernion, J. B., Verma, I. M., and Cline, M. J., Expression of cellular oncogenes in human malignancies, *Science*, 224, 256, 1984.
28. Gross, L., *Oncogenic Viruses*, Pergamon Press, New York, 1983, 103.
29. Vitetta, E. S., Krolick, K. A., Miyama-Inaba, M., Cushley, W., and Uhr, J. W., Immunotoxins: a new approach to cancer therapy, *Science*, 219, 644, 1983.

30. Bjorkland, B. and Bjorkland, V., Antigenicity of pooled human malignant and normal tissues by cyto-immunological technique: presence of an insoluble, heat-labile tumor antigen, *Int. Arch. Allergy Appl. Immunol.*, 10, 153, 1957.
31. Nathrath, W. B. J., Heidenkummer, P., Arnholdt, H., Bassermann, R., Lohrs, U., Pemanetter, W., Remberger, K., and Wiebecke, B., Distribution of tissue polypeptide antigen in normal and neoplastic tissues, *Protides Biol. Fluids*, 31, 437, 1983.
32. Bjorkland, B., Overview of TPA: specificity, immunochemistry, and biological basis, *Protides Biol. Fluids*, 31, 425, 1983.
33. Nemoto, T., Constantine, R., and Chu, T. M., Human tissue polypeptide antigen in breast cancer, *JNCI*, 63, 1347, 1979.
34. Ashall, F., Bramwell, M. E., and Harris, H., A new marker for human cancer cells. The Ca antigen and the Ca1 antibody, *Lancet*, 2, 1, 1982.
35. Woodhouse, C. S., Seiler, C., Morgan, A. C., and Oldham, R. K., Immunohistochemical detection of the Ca antigen in normal and tumor tissues of humans by use of Ca1 monoclonal antibody, *JNCI*, 74, 383, 1985.
36. Hanisch, F.-G., Uhlenbruck, G., and Dienst, C., Structure of tumor-associated carbohydrate antigen Ca 19-9 on human seminal-plasma glycoproteins from healthy donors, *Eur. J. Biochem.*, 144, 467, 1984.
37. Bolmer, S. D. and Davidson, E. A., Preparation and properties of a glycoprotein associated with malignancy, *Biochemistry*, 20, 1047, 1981.
38. Brewer, L. M., Durkin, J. P., and MacManus, J. P., Immunocytochemical detection of oncomodulin in tumor tissue, *J. Histochem. Cytochem.*, 32, 1009, 1984.
39. MacManus, J. P., Brewer, L. M., and Whitfield, J. F., The potential of the calcium-binding protein, oncomodulin, as a tumor marker, *Protides Biol. Fluids*, 31, 399, 1983.
40. Bogoch, S., Bogoch, E. S., Antich, P., Dungan, S. M., Harris, J. H., Ambrus, J. L., and Powers, N., Elevated levels of antimalignin antibody are quantitatively related to longer survival in cancer patients, *Protides Biol. Fluids*, 31, 739, 1983.
41. Bhattacharya, M., Chatterjee, S. K., and Barlow, J. J., Identification of a human cancer-associated antigen defined with monoclonal antibody, *Cancer Res.*, 44, 4528, 1984.
42. Bhavanandan, V. P. and Davidson, E. A., Cell surface glycoprotein markers for neoplasia, *Methods Cancer Res.*, 19, 53, 1982.
43. Kennett, R. H., Jonak, Z. L., and Byrd, R., Cell surface changes in malignancy, *Methods Cancer Res.*, 20, 355, 1982.
44. Hakomori, S., Glycosphinolipids as differentiation and tumor markers and as regulators of cell proliferation, in *Molecular Biology of Tumor Cells*, Wahren, B. et al., Eds., Raven Press, New York, 1985, 139.
45. Hakomori, S., Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives, *Cancer Res.*, 45, 2405, 1985.
46. Feizi, T., Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens, *Nature (London)*, 314, 53, 1985.
47. Black, P. H., Hakomori, S.-I., and Warren, L., Sapporo Cancer Seminar: membrane-associated alterations in cancer-biochemical strategies against cancer, *Cancer Res.*, 43, 2322, 1983.
48. Smets, L. A. and Van Beek, W. P., Carbohydrates of the tumor cell surface, *Biochim. Biophys. Acta*, 738, 237, 1984.
49. Warren, L., Buck, C. A., and Tuszynski, G. P., Glycopeptide changes and malignant transformation. A possible role of carbohydrate in malignant behavior, *Biochim. Biophys. Acta*, 516, 97, 1978.
50. Bast, R., Jr., Klug, T., St. John, É., Jenison, E., Niloff, J., Lazarus, H., Berkowitz, R., Leavitt, T., Griffiths, T. et al., A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer, *N. Engl. J. Med.*, 309, 883, 1983.
51. Bhavanandan, V. P., Kemper, J. G., and Bystyn, J. C., Publication and partial characterization of a murine melanoma-associated antigen, *J. Biol. Chem.*, 255, 5145, 1980.
52. Bramwell, M. E., Bhavanandan, V. P., Wiseman, G., and Harris, H., Structure and function of the Ca antigen, *Br. J. Cancer*, 48, 177, 1983.
53. Lan, M. S., Finn, O. J., Fernsten, P. D., and Metzbar, R. S., Isolation and properties of a human pancreatic adenocarcinoma-associated antigen DU-PAN-2, *Cancer Res.*, 45, 305, 1985.
54. Nodelman, E., Hakomori, S., Kannagi, R., Levery, S., Yeh, M. Y., Hellstrom, K. E., and Hellstrom, I., Characterization of a human melanoma-associated ganglioside antigen defined by a monoclonal antibody, *J. Biol. Chem.*, 257, 12752, 1982.
55. Pukel, C. S., Lloyd, K. O., Trabassos, L. R., Dippold, W. G., Oettgen, J. F., Quinones, R. R., Youle, R. J., and Kersey, J. H., Anti-T cell monoclonal antibodies conjugated to ricin as potential reagents for human GvHD prophylaxis: effect on the generation of cytotoxic T cells in both peripheral blood and bone marrow, *J. Immunol.*, 132, 678, 1984.

56. Siddiqui, B., Buehler, J., De Gregorio, M. W., and Macher, B., Differential expression of ganglioside GD3 by human leukocytes and leukemia cells, *Cancer Res.*, 44, 5262, 1984.
57. Cahan, L. D., Irie, R. I., Singh, R., and Cassidenti, A., Identification of human neuroectodermal tumor antigen (OFA-1-1) as ganglioside GD2, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 5392, 1982.
58. Kniep, B., Monner, D. A., Burrichtter, H., Diehl, V., and Muhlradt, P. F., Gangliotriaosylceramide (asialo GM2), a glycosphingolipid marker for cell lines from patients with Hodgkin's disease, *J. Immunol.*, 131, 1591, 1983.
59. Nudelman, E., Kannagi, R., Hakomori, S., Parsons, M., Lipinski, M., Wiels, J., Fellous, M., and Tursz, T., A glycolipid antigen associated with Burkit lymphoma defined by a monoclonal antibody, *Science*, 220, 509, 1983.
60. Falk, K. E., Karlsson, K. A., Larson, G., Thurin, J., Blaszcyk, M., Steplewski, Z., and Koprowski, H., Mass spectrometry of a human tumor glycolipid antigen being defined by mouse monoclonal antibody NS-19-9, *Biochem. Biophys. Res. Commun.*, 100, 383, 1983.
61. Bukushi, Y., Hakomori, S., Nudelman, E., and Cochran, N., Selective siolation of hybridoma antibodies that differentially recognize mono-, di-, and trifucosylated type 2 chain, *J. Biol. Chem.*, 259, 4681, 1984.
62. Hakomori, S., Nudelman, E., Levery, S. B., and Kannagi, R., Novel fucolipids accumulating in human adenocarcinoma. I. Glycolipids with di- or trifucosylated type 2 chain, *J. Biol. Chem.*, 259, 4672, 1984.
63. Koprowski, H., Herlyn, M., Steplewski, Z., and Sears, H. F., Specific antigen in serum of patients with colon carcinoma, *Science*, 212, 53, 1981.
64. Magnani, J. L., Nilsson, B., Brockhaus, M., Zopr, D., Steplewski, Z., Koprowski, H., and Ginsburg, V., A monoclonal antibody defined associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-N-fucopentaose. II, *J. Biol. Chem.*, 257, 14365, 1982.
65. Bremer, E. G., Levery, A. B., Sonnino, S., Ghidoni, R., Canevari, S., Kannagi, R., and Hakomori, S., Characterization of a glycosphingolipid antigen defined by the monoclonal antibody MBrl expressed in normal and neoplastic epithelial cells of human mammary gland, *J. Biol. Chem.*, 259, 14773, 1984.
66. Yamashito, K., Hitoi, A., Tsuchda, Y., Nishi, S., and Kobata, A. A., A comparative study of the sugar chains of alpha glutamyl transferase purified from rat liver and rat AH-66 hepatoma cells, *Cancer Res.*, 43, 5059, 1983.
67. Shinitzky, M., Membrane fluidity in malignancy: adversative and recuperative, *Biochim. Biophys. Acta*, 738, 251, 1984.
68. Hakomori, S., Blood group glycolipid antigens and their modifications as human cancer antigens, *Am. J. Clin. Pathol.*, 82, 635, 1984.
69. Hakomori, S. and Kobata, A., Blood group antigens, in *The Antigens*, Vol. 2, Sela, M., Ed., Academic Press, New York, 1974, 80.
70. Watanabe, K. and Hakomori, S., Status of blood group carbohydrate chains in ontogenesis and oncogenesis, *J. Exp. Med.*, 144, 644, 1976.
71. Springer, G. F., T and Tn general carcinoma autoantigens, *Science*, 224, 1198, 1984.
72. Zabel, P. L., Noujaim, A. A., Shysh, A., and Bray, J., Radioiodinated peanut lectin: a potential radiopharmaceutical for immunodetection of carcinoma expressing the T-antigen, *Eur. J. Nucl. Med.*, 8, 250, 1983.
73. Hakomori, S. and Kannagi, R., Glycospingolipids as tumor associated differentiation markers, *JNCI*, 71, 231, 1983.
74. Blasczyk, M., Hansson, G. C., Karlsson, K.-A., Larson, G., Stromberg, N., Thurin, J., Herlyn, M., Steplewski, Z., and Koprowski, H., Lewis blood group antigens defined by monoclonal anti-codon carcinoma antibodies, *Arch. Biochem. Biophys.*, 233, 161, 1984.
75. Kannagi, R., Papayannopoulou, T., Nakamoto, B. et al., Carbohydrate antigen profiles of human erythroleukemia cell lines HEL and K562, *Blood*, 62, 1230, 1983.
76. Hakkinen, I., A-like blood group antigen in gastric cancer cells of patients in blood groups O or B, *JNCI*, 44, 1183, 1970.
77. Hakomori, S., Wang, S. M., and Young, W. W., Isoantigenic expression of Forssman glycolipid in human gastric and colonic mucosa: its possible identity with "A-like antigen" in human cancer, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3023, 1977.
78. Weils, J., Fellous, M., and Turszi, T., Monoclonal antibody against a Burkitt lymphoma associated antigen, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 6485, 1981.
79. Banjo, C., Shuster, J., and Gold, P., Intermolecular heterogeneity of the carcinoembryonic antigen, *Cancer Res.*, 34, 2114, 1974.
80. Chandrasekaran, E. V., Davila, M., Nixon, D. W., Goldfarb, M., and Mendicino, J., Isolation and structures of the oligosaccharide units of carcinoembryonic antigen, *J. Biol. Chem.*, 258, 7213, 1983.

81. Yeoman, L. C., Taylor, C. W., and Charabarty, S., Human colon tumor antigens, *Methods Cancer Res.*, 19, 234, 1982.
82. Hirai, H., Model systems of AFP and CEA expression, *Methods Cancer Res.*, 18, 39, 1979.
83. Accolla, R. S., Carrel, S., Phan, M., Heumann, D., and Mach, J. P., First report of the production of somatic cell hybrids secreting monoclonal antibodies specific for carcinoembryonic antigen (CEA), *Protides Biol. Fluids*, 27, 31, 1979.
84. Grunert, F., Wank, K., Luckenback, G. A., and Von Kleist, S., Monoclonal antibodies against CEA. Comparison of the immunoprecipitates by fingerprint analysis, *Oncodevel. Biol. Med.*, 3, 191, 1982.
85. Hedin, A., Hammarstrom, S., and Larsson, A., Specificities and binding properties of eight monoclonal antibodies against carcinoembryonic antigen, *Mol. Immunol.*, 19, 1641, 1982.
86. Primus, F. J., Newell, K. D., Blue, A., and Goldenberg, D. M., Immunological heterogeneity of carcinoembryonic antigen: immunohistochemical detection of carcinoembryonic antigen determinants in colonic tumors with monoclonal antibodies, *Cancer Res.*, 43, 693, 1983.
87. Colcher, D., Hand, P. H., Nuti, M., and Schlom, J., Differential binding to human mammary and nonmammary tumors of monoclonal antibodies reactive with carcinoembryonic antigen, *Cancer Invest.*, 1, 127, 1983.
88. Kupchik, H. Z., Zurawski, V. R., Hurdell, J. G., Zamchek, N., and Black, P., Monoclonal antibodies to carcinoembryonic antigen produced by somatic cell fusion, *Cancer Res.*, 41, 3306, 1981.
89. Rogers, G. T., Rawlins, G. A., and Bagshawe, K. D., Somatic-cell hybrids producing antibodies against CEA, *Br. J. Cancer*, 43, 1, 1981.
90. Hedin, A., Wahren, B., and Hammerstrom, S., Tumor localization of CEA-containing human tumors in nude mice by means of monoclonal anti-CEA antibodies, *Int. J. Cancer*, 30, 547, 1982.
91. Goldenberg, D. M., Gaffar, S. A., Bennett, S. J., and Beach, J. L., Experimental radioimmunotherapy of a xenografted human colonic tumor (GW-39) producing carcinoembryonic antigen, *Cancer Res.*, 41, 4354, 1981.
92. Dillman, R. O., Beauregard, J. C., Sobol, R. E., Royston, I., Bartholomew, R. M., Hagan, P. S., and Halpern, S. E., Lack of radioimmunodetection and complications associated with monoclonal anticarcinoembryonic antigen antibody cross-reactivity with antigen on circulating cells, *Cancer Res.*, 44, 2213, 1984.
93. DeLand, F. H. and Goldenberg, D. M., In vivo cancer diagnosis by radioimmunodetection, in *Radioimmunoimaging and Radioimmunotherapy*, Burchiel, S. W. and Rhodes, B. A., Eds., Elsevier, New York, 1983, 329.
94. Sell, S., Monoclonal antibodies to alphafetoprotein and regulation of AFP gene expression, in *Monoclonal Antibodies in Cancer*, Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N. J., 1985, chap. 3.
95. Micheel, B., Fiebach, H., Karsten, U., Gousseu, A. et al., Monoclonal antibodies to different epitopes of human alpha-fetoprotein (AFP), *Eur. J. Cancer Clin. Oncol.*, 19, 1239, 1983.
96. Bellet, D., Arrang, J. M., Contesso, G., Cailaud, J. M., and Bohuon, C., Localization of the beta subunit of human chorionic gonadotropin on various tumors, *Cancer*, 16, 433, 1980.
97. Griffing, G. and Vaitukaitis, J. L., Hormone-secreting tumors, in *Cancer Markers*, Sell, S., Ed., Humana Press, Clifton, N.J., 1980, 169.
98. Matsuura, S., Ohashi, M., Chen, H. C., and Hodgen, G. D., A human chorionic gonadotropin-specific antiserum against synthetic peptide analogs to the carboxyl-terminal peptide of its beta-subunit, *Endocrinology*, 104, 396, 1979.
99. Ehrlich, P. H., Mousstafa, Z. A., Krichevsky, A., and Mesa-Tejada, R., Human chronic gonadotropin detection with monoclonal antibodies, in *Monoclonal Antibodies in Cancer*, Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N.J., 1985, chap. 4.
100. Birken, S., Fetherston, J., Canfield, R., and Boime, I., The amino acid sequences of the prepeptides contained in the alpha and beta subunits of human choriogonadotropin, *J. Biol. Chem.*, 256, 1816, 1981.
101. Horne, C. H. W., Reid, I. N., and Milne, G. D., Prognostic significance of inappropriate production of pregnancy proteins by breast cancers, *Lancet*, 2, 279, 1976.
102. Saxena, B. N., Goldstein, D. P., Emerson, K., and Selenkow, H. A., Serum placental lactogen levels in patients with molar pregnancy and trophoblastic tumors, *Am. J. Obstet. Gynecol.*, 102, 115, 1968.
103. Seppala, M., Rutane, E.-M., Heikinheimo, M., Jalanko, H., and Engvall, E., Detection of trophoblastic tumour activity by pregnancy-specific beta-1-glycoprotein, *Int. J. Cancer*, 21, 165, 1978.
104. Choe, B. and Rose, N. R., Prostatic acid phosphatase: a marker for human prostatic adenocarcinoma, *Methods Cancer Res.*, 19, 199, 1982.
105. Balinsky, D., Enzymes and isoenzymes in cancer, in *Cancer Markers*, Sell, S., Ed., Humana Press, Clifton, N.J., 1982, 191.
106. Fishman, W. H., Inglis, N. R., Vaitukaitis, J., and Stolbach, L. L., Regan isoenzyme and human chorionic gonadotropin in ovarian cancer, *Natl. Cancer Inst. Monogr.*, 42, 63, 1975.

107. Tsukazaki, K., Hayman, E. G., and Ruoslahti, E., Effects of ricin A chain conjugates of monoclonal antibodies to human alpha-fetoprotein and placental alkaline phosphatase on antigen-producing tumor cells in culture, *Cancer Res.*, 45, 1834, 1985.
108. Blackstock, R. and Humphrey, B., Cell surface markers in the characterization of leukemias, *Methods Cancer Res.*, 19, 3, 1982.
109. Reinherz, E. L., Kung, P. C., Goldstein, G., Levey, R. H., and Schlossman, S. F., Discrete stages of human intrathymic differentiation. Analysis of normal thymocytes and leukemic lymphoblasts of T-cell linage, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 1588, 1980.
110. Reinherz, E. L. and Schlossman, S. F., The differentiation and function of human lymphocytes, *Cell,* 19, 821, 1980.
111. Lampson, L. and Levy, R., Two populations of Ia-like molecules on a human B cell line, *J. Immunol.*, 125, 293, 1980.
112. Moretta, L., Mingari, M. C., Moretta, A., and Fauci, A. S., Human lymphocyte surface markers, *Semin. Hematol.*, 19, 273, 1982.
113. Calvert, J. E., Maruyama, S., Tedder, T. F., Webb, C. F., and Cooper, M. D., Cellular events in the differentiation of antibody-secreting cells, *Semin. Hematol.*, 21, 226, 1984.
114. Ceredig, R., Lopez-Botet, M., and Moretta, L., Phenotypic and functional properties of mouse and human thymocytes, *Semin. Hematol.*, 21, 244, 1984.
115. Moretta, A., Pantelo, G., Maggi, E., and Mingari, M., Recent advances in the phenotypic and functional analysis of human T lymphocytes, *Semin. Hematol.*, 21, 257, 1984.
116. Caligaris-Cappio, F. and Janossy, G., Surface markers in chronic lymphoid leukemia of B cell type, *Semin. Hematol.*, 22, 1, 1985.
117. Harden, E. A. and Haynes, B. F., Phenotypic and functional characterization of human malignant T cells, *Semin. Hematol.*, 22, 13, 1985.
118. Ritz, J. and Schlossman, S. F., Utilization of monoclonal antibodies in the treatment of leukemia and lymphoma, *Blood,* 59, 1, 1982.
119. Andrews, R. G. and Bernstein, I. D., Nonlymphoblastic leukemia-associated antigens identified by monoclonal antibodies, in *Monoclonal Antibodies in Cancer,* Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N.J., 1985, 167.
120. Naito, K., Knowels, R., and Real, F., Analysis of two new leukemia associated antigens detected on human T acute lymphoblastic leukemia using monoclonal antibodies, *Blood,* 62, 852, 1983.
121. Negoro, S. and Seon, B. K., Several new monoclonal antibodies directed to human T-cell leukemia antigens, *Cancer Res.*, 42, 4259, 1982.
122. Berger, C., Morrison, S., and Chu, A., Diagnosis of cutaneous T cell lymphoma by use of monoclonal antibodies reactive with tumor associated antigens, *J. Clin. Invest.*, 70, 1205, 1982.
123. Robert-Guroff, M. and Shepard, E., A monoclonal antibody specific for a 52,000-molecular-weight human T-cell leukemia virus-associated glycoprotein expressed by infected cells, *J. Virol.*, 53, 214, 1985.
124. Sell, S. and Reisfeld, R. A., Eds., *Monoclonal Antibodies in Cancer,* Humana Press, Clifton, N.J., 1985.
125. Wright, G. L., Ed., *Monoclonal Antibodies and Cancer,* Marcel Dekker, New York, 1985.
126. Sulitzeanu, D., Human cancer-associated antigens: present status and implications for immunodiagnosis, *Adv. Cancer Res.*, 44, 1, 1985.
127. Price, M. R., Hannant, D., Embleton, M. J., and Baldwin, R. W., Icrew workshop report: detection and isolation of tumor-associated antigens, *Br. J. Cancer,* 41, 843, 1980.
128. Ghose, T., Norvell, S. T., Guclu, A., and MacDonald, A. S., Immunochemotherapy of human malignant melanoma with chlorambucil-carrying antibody, *Eur. J. Cancer,* 11, 321, 1975.
129. Ross, A. H., Herlyn, M., Ernest, C. S., Guerry, D., Bennicelli, J., Ghrist, B. F. D., Atkinson, B., and Koprowski, H., Immunoassay for melanoma-associated proteoglycan in the sera of patients using monoclonal and polyclonal antibodies, *Cancer Res.*, 44, 4647, 1984.
130. Reisfeld, R. A., Monoclonal antibodies as probes for the molecular structure and biological function of melanoma-associated antigens, in *Monoclonal Antibodies in Cancer,* Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N.J., 1985, 205.
131. Natali, P. G., Bigotti, A., Cavaliere, R., Nicotra, M. R., and Ferrone, S., Phenotyping of lesions of melanocyte origin with monoclonal antibodies to melanoma-associated antigens to HLA antigens, *JNCI,* 73, 13, 1984.
132. Khosravi, M. J., Dent, P. B., and Liao, S. K., Structural characterization and biosynthesis of gp87, a melanoma associated oncofetal antigen defined by monoclonal antibody 142,240, *Int. J. Cancer,* 35, 73, 1985.
133. Reisfeld, R. A. and Ferrone, S., Eds., *Melanoma Antigens and Antibodies,* Plenum Press, New York, 1982.

134. Dippold, W. G., Lloyd, K. O., Houghton, A. N., Li, L. T. C., Ikeda, H., Oettgen, H. F., and Old, L. J., Human melanoma antigens defined by monoclonal antibodies, in *Hybridomas in Cancer Diagnosis and Treatment,* Mitchell, M. S. and Dettgen, H. F., Eds., Raven Press, N.Y., 1982, 173.
135. Carrel, S., deTribolet, N., and Mach, J. P., Human melanoma and glioma associated antigen(s) identified by monoclonal antibodies, *Methods Cancer Res.,* 20, 318, 1982.
136. Brown, J. P., Hewick, R. M., Hellstrom, I., Hellstrom, K. E., Doolittle, R. F., and Dreyer, W. J., Human melanoma-associated antigen p97 is structurally and functionally related to transferrin, *Nature (London),* 296, 171, 1982.
137. Koprowski, H. and Herlyn, M., Human tumor antigens, in *Molecular Biology of Tumor Cells,* Wahren, B. et al., Eds., Raven Press, New York, 1985, 123.
138. Cheresh, D. A., Varki, A. P., Varki, N. M., Stallcup, W. B., Levine, J., and Reisfeld, R. A., A monoclonal antibody recognizes an O-acylated sialic acid in a human melanoma-associated ganglioside, *J. Biol. Chem.,* 259, 7453, 1984.
139. Ghose, T., Ferrone, S., Imai, K., Norvell, S. T., Jr., Luner, S. J., Martin, R. H., and Blair, A. H., Imaging of human melanoma xenografts in nude mice with a radiolabelled monoclonal antibody, *JNCI,* 69, 823, 1982.
140. Murray, J. L., Rosenblum, M. G., Sobol, R. E., Bartholomew, R. M., Plager, C. E., Haynie, T. P., Johns, M. F., Glenn, H. J., Lamki, L., Benjamin, R. S., Papadopoulous, N., Boddie, A. W., Frincke, J. M., David, G. S., Carol, D. J., and Hersh, E. M., Radioimmunoimaging in malignant melanoma with ^{111}In-labeled monoclonal antibody 96.5, *Cancer Res.,* 45, 2376, 1985.
141. Schroff, R. W., Foon, K. A., Beatty, S. M., Oldham, R. K., and Morgan, A. C., Jr., Human antimurine immunoglobulin responses in patients receiving monoclonal antibody therapy, *Cancer Res.,* 45, 879, 1985.
142. Larson, S. M., Brown, J. P., Wright, P. W., Carrasquillo, J. A., Hellstrom, S., and Hellstrom, K. E., Imaging of melanoma with I-131-labelled monoclonal antibodies, *J. Nucl. Med.,* 24, 123, 1983.
143. Larson, S. M., Carrasquillo, J. A., Krohn, K. A., Brown, J. P., McGuffin, R. W., Ferens, J. M., Graham, M. M., Hill, L. D., and Beaumier, P. L., Localization of 131-I-labeled P97-specific Fab fragments in human melanoma as a basis for radiotherapy, *J. Clin. Invest.,* 72, 2101, 1983.
144. Ghose, T., Kulkarni, P. N., Ferrone, S., Blair, A. H., Mammen, M., and Sadi, D., Inhibition of human melanoma M21 xenografts in nude mice by methotrexate to monoclonal and polyclonal antimelanoma antibodies, submitted.
145. Irie, R. F., Sze, L. L., and Saxton, R. E., Human antibody to OFA-I, a tumor antigen, produced in vitro by Epstein-Barr virus-transformed human B lymphoid cell lines, *Proc. Natl. Acad. Sci. U.S.A.,* 79, 5666, 1982.
146. Goldenberg, D. M., Deland, F., Kim, E., Bennett, S., Primus, F. J., Van Nagell, J. R., Estes, N., DeSimone, P., and Rayburn, P., Use of radiolabeled antibodies to carcinoembryonic antigen for the detection and localization of diverse cancers by external photoscanning, *N. Engl. J. Med.,* 298, 1384, 1978.
147. Mach, J.-P., Carrel, S., Forni, M., Ritschard, J., Donath, A., and Alberto, P., Tumor localization of radiolabeled antibodies against carcinoembryonic antigen in patients with carcinoma, *Cancer Res.,* 43, 5593, 1983.
148. Gold, D. U. and Goldenberg, D. M., Antigens associated with human solid tumors, in *Cancer Markers,* Sell, S., Ed., Humana Press, Clifton, N.J., 1982, 329.
149. Bara, J., Paul-Gardais, A., Loisillier, F., and Burton, P., Isolation of a sulfated glycopepetidic antigen from human gastric tumors. Its localization in normal and cancerous gastrointestinal tissues, *Int. J. Cancer,* 21, 133, 1978.
150. Hattori, H., Uemura, K., and Taketomi, T., Glycolipids of gastric cancer. The presence of blood group A-active glycolipids in cancer tissues from blood group O patients, *Biochim. Biophys. Acta,* 666, 361, 1981.
151. Smith, J. B. and O'Neill, R. T., Fetal gut antigen: a substance in fetal gut and its relationship to gut carcinoma, *Res. Commun. Chem. Pathol. Pharmacol.,* 2, 1, 1971.
152. Pusztaszeri, G., Saravis, C. A., and Zamcheck, N., The zinc glycinate marker in human colon carcinoma, *JNCI,* 56, 275, 1976.
153. Pant, K. D., Dahlman, H. L., and Goldenberg, D. M., Further characterization of CSAp: an antigen associated with gastrointestinal and ovarian tumors, *Cancer,* 42, 230, 1978.
154. Gaffar, S. A., Pant, K. D., Shochat, D., Bennett, S. J., and Goldenberg, D. M., Experimental studies of tumor radioimmunodirection using antibody mixtures against carcinoembryonic antigen (CEA) and colon-specific antigen-p (CSAp), *Int. J. Cancer,* 27, 101, 1981.
155. Steplewski, Z. and Koprowski, H., Monoclonal antibody development in the study of colorectal carcinoma-associated antigen, *Methods Cancer Res.,* 20, 285, 1982.
156. Chatal, J.-F., Saccavini, J.-C., Fumoleau, P., Douillard, J.-Y., Curtet, C., Kremer, M., LeMevel, B., and Koprowski, H., Immunoscintigraphy of colon carcinoma, *J. Nucl. Med.,* 25, 307, 1984.

157. Blumberg, B. B. and London, W. T., Hepatitis B virus and the prevention of primary cancer of the liver, *JNCI*, 74, 267, 1985.
158. Ona, F. V., Zamcheck, N., Dhar, P., Moore, T., and Kupchick, H. Z., Carcinoembryonic antigen (CEA) in the diagnosis of pancreatic cancer, *Cancer*, 31, 324, 1972.
159. Banwo, O., Versey, J., and Hobbs, J. R., New oncofetal antigen for human pancreas, *Lancet*, 1, 643, 1974.
160. Gelder, F. B., Reese, C. J., Moossa, A. R., Hall, T., and Hunter, R., Purification, partial characterization and clinical evaluation of pancreatic oncofetal antigen, *Cancer Res.*, 38, 313, 1978.
161. Loor, R., Kuriyama, M., Bodziak, M. L. M., Inaji, H., Douglass, H. O., Jr., Berjian, R., Nicolai, J. J. H., Tytgat, G. N., and Chu, T. M., Simultaneous evaluation of a pancreas-specific antigen and a pancreatic cancer-associated antigen in pancreatic carcinoma, *Cancer Res.*, 44, 3604, 1984.
162. Gold, D. V., Hollingsworth, P., Kremer, T., and Nelson, D., Identification of a human pancreatic duct tissue-specific antigen, *Cancer Res.*, 43, 235, 1983.
163. Chu, T. M., Monoclonal antibodies to human prostate cancer-related antigens, in *Monoclonal Antibodies in Cancer*, Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N.J., 1985, 309.
164. Metzgar, R. S., Gaillard, M. T., Levine, S. J., Tuck, F. L., Bossen, E. H., and Borowitz, M. J., Antigens of human pancreatic adenocarcinoma cells defined by murine monoclonal antibodies, *Cancer Res.*, 42, 601, 1982.
165. Parsa, I., Identification of human acinar cell carcinoma by monoclonal antibody and in vitro differentiation, *Cancer Lett.*, 15, 115, 1982.
166. Parsa, I., Sutton, A. L., Chen, C. K., and Delbridge, C., Monoclonal antibody for identification of human duct cell carcinoma of pancreas, *Cancer Lett.*, 17, 217, 1982.
167. Chin, J. and Miller, F., Identification and localization of human pancreatic tumor-associated antigens by monoclonal antibodies to RWP-1 and RWP-2 cells, *Cancer Res.*, 45, 1723, 1985.
168. Schmiegel, W. H., Kalthoff, H., Arndt, R., Gieseking, J., Greten, H., Kloppel, G., Kreiker, C., Ladak, A., Lampe, V., and Ulrich, S., Monoclonal antibody-defined human pancreatic cancer-associated antigens, *Cancer Res.*, 45, 1402, 1985.
169. Metzgar, R. S., Rodriguez, N., Finn, O, J., Lan, M. S., Daasch, V. N., Fernsten, P. D., Meyers, W. C., Saindelar, W., Sandler, R. S., and Seigler, H. F., Detection of a pancreatic cancer-associated antigen (DU-PAN-2 antigen) in serum and ascites of patients with adenocarcinoma, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 5242, 1984.
170. Imam, A., Improvement in the immunological method for the detection of human breast carcinoma, *Oncology*, 39, 255, 1982.
171. Schwartz, M. K., Estrogen receptors and tumor associated antigens in breast cancer, *Ann. Clin. Lab. Sci.*, 9, 258, 1979.
172. Imam, A., Drushella, M. M., Taylor, C. R., and Tokes, Z. A., Generation and immunohistological characterization of human monoclonal antibodies to mammary carcinoma cells, *Cancer Res.*, 45, 263, 1985.
173. Schlom, J., Colcher, F., Horan Hand, P., Greiner, J., Wunderlich, D., Weeks, M., Fisher, P. B., Noguchi, P., Pestka, S., and Kufe, D., Monoclonal antibodies reactive with breast tumor-associated antigens, *Adv. Cancer Res.*, 43, 143, 1985.
174. Colcher, D., Hand, H., Nuti, M., and Schlom, J., A spectrum of monoclonal antibodies reactive with human mammary tumor cells, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 3199, 1981.
175. Teramoto, Y. A., Mariani, R., Wunderlich, D., and Schlom, J., The immunohistochemical reactivity of a human monoclonal antibody with tissue sections of human mammary tumors, *Cancer*, 50, 241, 1982.
176. Canevari, S., Fossati, G., Balsari, A., Sonnino, S., and Colmaghi, M. I., Immunochemical analysis of the determinant recognized by a monoclonal antibody (MBr1) which specifically binds to human mammary epithelial cells, *Cancer Res.*, 43, 1301, 1983.
177. Deland, F. H. and Goldenberg, D. M., Diagnosis and treatment of neoplasms with radionuclide-labeled antibodies, *Semin. Nucl. Med.*, 15, 2, 1985.
178. Goldenberg, D. M., Kim, E. E., and Deland, F. H., Human chorionic gonadotrophin radioantibodies in the radioimmunodetection of cancer and for disclosure of occult metastases, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 7754, 1981.
179. Wang, M. C., Kuriyama, M., Papsidero, L. D., Loor, R. M., Valenzuela, L. A., Murphy, G. P., and Chu, T. M., Prostate antigen of human cancer patients, *Methods Cancer Res.*, 19, 179, 1982.
180. Ware, J. L., Paulson, D. F., Parks, S. F., and Webb, K. S., Production of monoclonal antibody alpha pro 3 recognizing a human prostatic carcinoma antigen, *Cancer Res.*, 42, 1215, 1982.
181. Stein, B. S., Vangore, S., and Petersen, R. O., Immunoperoxidase localization of prostatic antigens: comparison of primary and metastatic sites, *Urology*, 24, 146, 1984.

182. Starling, J. J., Sleg, S. M., Beckett, M. L., Schellhammer, P. T., La Daga, L. E., and Wright, G. L., Monoclonal antibodies to human prostate and bladder tumor associated antigens, *Cancer Res.*, 42, 3084, 1982.
183. Lindgren, J., Pak, K. Y., Ernst, C., Rovera, G., Steplewski, Z., and Kiprowski, H., Shared antigens of human prostate cancer cell lines as defined by monoclonal antibodies, *Hybridoma*, 4, 37, 1985.
184. Frankel, A. E., Roose, R. V., Wang, M. C., Chu, T. M., and Herzenberg, L. A., Monoclonal antibodies to a human prostate antigen, *Cancer Res.*, 42, 3714, 1982.
185. Lee, C. L., Li, C. Y., Jou, Y. H., Murphy, G. P., and Chu, T. M., Immunochemical characterization of prostatic acid phosphatase with monoclonal antibodies, *Ann. N.Y. Acad. Sci.*, 390, 52, 1982.
186. Webb, K. S., Ware, J. L., Parks, S. F., Briter, W. H., and Paulson, D. F., Monoclonal antibodies to different epitopes of a prostate tumor associated antigen: implications for immunotherapy, *Cancer Immunol. Immunother.*, 14, 155, 1983.
187. Starling, J. J. and Wright, G. L., Jr., Disulfide bonding of a human prostate tumor-associated membrane antigen recognized by monoclonal antibody D83.21, *Cancer Res.*, 45, 804, 1985.
188. Lee, C. L., Kawinski, E., Leong, S. S., Horoseziewicz, J. S., Murphy, G. P., and Chu, M., Radioimmunodetection and immunochemotherapy of xenografted human prostatic tumors using monoclonal antibody, *Fed. Proc.*, 43, 682, 1983.
189. Kuriyama, M., Wang, M. C., Lee, C. L., Killian, C. S., Papsidero, L. D., Inaji, H., Loor, R. M., Lin, M. F., Nishiura, T., Slack, N. H., and Murphy, G. P., Multiple marker evaluation in human prostate cancer with the use of tissue specific antigens, *JNCI*, 68, 99, 1982.
190. Bast, R. C., Jr. and Knapp, R. C., Immunologic approaches to the management of ovarian carcinoma, *Semin. Oncol.*, 11, 264, 1984.
191. Bhattacharya, M. and Barlow, J. J., Tumor markers of ovarian cancer, *Int. Adv. Surg. Oncol.*, 2, 155, 1979.
192. Bhattacharya, M., Chatterjee, S. K., Barlow, J. J., and Fuji, H., Monoclonal antibodies recognizing tumor-associated antigen of human ovarian mucinous cystadenocarcinomas, *Cancer Res.*, 42, 1650, 1982.
193. Knauf, S. and Urbach, G. I., Study of ovarian cancer patients using a radioimmunoassay for human ovarian tumor associated antigen OCA, *Am. J. Obstet. Gynecol.*, 138, 1222, 1980.
194. Bast, R. C., Feeney, M., Lazarus, H., Nadler, L. M., Colvin, R. B., and Knapp, R. C., Reactivity of a monoclonal antibody with human ovarian carcinoma, *J. Clin. Invest.*, 68, 1331, 1981.
195. Tagliabue, E., Menard, S., Torre, G. D., Baranti, P., Costantini, R. M., Porro, G., and Colnaghi, M. I., Generation of monoclonal antibodies reacting with human epithelial ovarian cancer, *Cancer Res.*, 45, 379, 1985.
196. Bizzari, J. P., Mackillop, W. J., and Buick, R. N., Cellular specificity of NB70K, a putative human ovarian tumor antigen, *Cancer Res.*, 43, 864, 1983.
197. Canney, P. A., Moore, M., Wilkinson, P. M., and James, R. D., Ovarian cancer antigen CA125: a prospective clinical assessment of its role as a tumour marker, *Br. J. Cancer*, 50, 765, 1984.
198. Van Nagall, J. R., Jr., Kim, E., Casper, S., Primus, J., Bennett, S., DeLand, F. H., and Goldenberg, D. M., Radioimmunodetection of primary and metastatic ovarian cancer using radiolabeled antibodies to carcinoembryonic antigen, *Cancer Res.*, 40, 502, 1980.
199. Epenetos, A. A., Mather, S., Granowska, M., Nimmon, C. C., Hawkins, L. R., Britton, K. E., Shepherd, J., Taylor-Papadimitriou, J., Durbin, H., and Malpas, J. S., Targeting of iodine-123-labelled tumour-associated monoclonal antibodies to ovarian, breast, and gastrointestinal tumours, *Lancet*, 2, 999, 1982.
200. Goldenberg, D. M., Kim, E. E., DeLand, F. H., Van Nagell, J. R., and Javadpour, N., Clinical radioimmunodetection of cancer with radioactive antibodies to human chorionic gonadotropin, *Science*, 208, 1284, 1980.
201. Order, S. E., Donahue, V., and Knapp, R. C., Immunotherapy of ovarian cancer, *Cancer*, 32, 573, 1973.
202. Symonds, E. M., Perkins, A. C., Pimm, M. V., Baldwin, R. W., Hardy, J. G., and Williams, D. A., Clinical implications for immunoscintigraphy in patients with ovarian malignancy: a preliminary study using monoclonal antibody 791T/36, *Br. J. Obstet. Gynecol.*, 92, 270, 1985.
203. Yachi, A., Matsuura, Y., Carpenter, C. M., and Hyde, L., Immunochemical studies on human lung cancer antigens soluble in 50% saturated ammonium sulfate, *JNCI*, 40, 663, 1968.
204. Braatz, J. A., Scharfe, T. R., Princler, G. L., and McIntire, K. R., Characterization of a human lung tumor-associated antigen and development of a radioimmunoassay, *Cancer Res.*, 42, 849, 1982.
205. Gazdar, A. F., Carney, D. N., and Minna, J. D., The biology of non-small cell lung cancer, *Semin. Oncol.*, 10, 3, 1983.
206. Mulshine, J. L., Cuttitta, F., Bibro, M., Fedorko, J., Fargion, S., Little, C., Carney, D. N., Gazdar, A. F., and Minna, J. D., Monoclonal antibodies that distinguish non-small cell from small cell lung cancer, *J. Immunol.*, 131, 1767, 1983.

207. Becker, K. I., Snider, R. H., Silva, O. L. et al., Calcitonin heterogeneity in lung cancer and medullary thyroid cancer, *Acta Endocrinol.*, 89, 89, 1978.
208. Cuttitta, F., Rosen, S., Gazdar, A. F., and Minna, J. D., Monoclonal antibodies that demonstrate specificity for several types of human lung cancer, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 4591, 1981.
209. Brenner, B. G., Jothy, S., Shuster, J., and Fuks, A., Monoclonal antibodies to human lung tumor antigens demonstrated by immunofluorescence and immunoprecipitation, *Cancer Res.*, 42, 3187, 1982.
210. Brown, D. T. and Moore, M., Monoclonal antibodies against two human lung carcinoma cell lines, *Br. J. Cancer*, 46, 794, 1982.
211. Kasai, M., Saxton, R. E., Holmes, E. C., Burk, M. W., and Morton, D. L., Membrane antigens detected on human lung carcinoma cells by hybridoma monoclonal antibody, *J. Surg. Res.*, 30, 403, 1981.
212. Harkozinska, A., Richter, R., Albert, Z., and Zawadzka, H., Antigenic heterogeneity of human lung cancer, *JNCI*, 70, 427, 1983.
213. Saji, S., Zylstra, S., Schepart, B. S., Ghosh, S. K., Jou, Y.-H., Takita, H., and Bankert, R., Monoclonal antibodies specific for two different histological types of human lung carcinoma, *Hybridoma*, 3, 119, 1983.
214. de Leij, L., Poppema, S., Nulend, J. K., ter Haar, A., Schwander, E., Ebbens, F., Postmus, P. E., and The, T. H., Neuroendocrine differentiation antigen on human lung carcinoma and kulchitski cells, *Cancer Res.*, 45, 2192, 1985.
215. Okabe, T., Kaizu, T., Ozawa, K., Urabe, A., and Takaku, F., Elimination of small cell lung cancer cells in vitro from human bone marrow by a monoclonal antibody, *Cancer Res.*, 45, 1930, 1985.
216. Bernal, S. D., Mabry, M., Stahel, R. A., Griffin, J. D., and Speak, J. A., Selective cytotoxicity of SM1 monoclonal antibody towards small cell carcinoma of the lung, *Cancer Res.*, 45, 1026, 1985.
217. Zimmer, A. M., Rosen, S. T., Spies, S. M., Polovina, M. R., Minna, J. D., Spies, W. C., and Silverstein, E. A., Radioimmunoimaging of human small cell lung carcinoma with I-131 tumor specific monoclonal antibody, *Hybridoma*, 4, 1, 1985.
218. Embleton, M. J., Monoclonal antibodies to esteogenic sarconoma antigens, in *Monoclonal Antibodies and Cancer*, Vol. 23, Wright, G. L., Jr., Ed., Marcel Dekker, New York, 1984, 181.
219. Price, M. R., Campbell, D. G., Robins, R. A., and Baldwin, R. W., Characteristics of a cell surface antigen defined by an anti-human osteogenic sarcoma monoclonal antibody, *Eur. J. Clin. Oncol.*, 19, 81, 1983.
220. Hosoi, S., Nakamura, T., Higashi, S., Yamamuro, T., Toyama, S., Shinomiya, K., and Mikawa, H., Detection of human osteosarcoma-associated antigen(s) by monoclonal antibodies, *Cancer Res.*, 42, 654, 1982.
221. Grofova, M., Forchhammer, J., Lizonova, A., and Popovic, M., Immunoprecipitation of membrane proteins of cultured human sarcoma cells, *Neoplasma*, 28, 633, 1981.
222. Nairn, R. C., Philip, J., Ghose, T., Porteous, I. B., and Fothergill, J. E., Production of a precipitin against renal cancer, *Br. Med. J.*, 1, 1702, 1963.
223. Ravitz, G., Watne, A. L., and Milam, D. F., Autoantibodies to human renal cell carcinoma, *J. Urol.*, 107, 26, 1972.
224. Ghose, T., Belitsky, P., Tai, J., and Janigan, D. T., Production and characterization of xenogeneic antisera to a human renal cell carcinoma associated antigen, *JNCI*, 63, 301, 1979.
225. Ghose, T., Norvell, S. T., Aquino, J., Belitsky, P., Tai, J., Guclu, A., and Blair, A. H., Localization of ^{131}I-labeled antibodies in human renal cell carcinomas & in a mouse hepatoma & correlation with tumor detection by photoscanning, *Cancer Res.*, 40, 3018, 1980.
226. Ueda, R., Ogata, S.-I., Morrissey, D. M., Finstad, C. L., Szkudlarek, J., Whitmore, W. F., Oettgen, H. F., Lloyd, K. O., and Old, L. J., Cell surface antigens of human renal cancer defined by mouse monoclonal antibodies: identification of tissue-specific kidney glycoproteins, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 5122, 1981.
226a. Luner, S. J., Ghose, T., Chatterji, S., Nolido-Cruz, H., and Belitsky, P., Monoclonal antibodies to kidney and tumor-associated surface antigens of human renal cell carcinoma, *Cancer Res.*, in press.
227. Scherberich, J., Hess, H., Alheid, U., Falkenberg, F., Mondorf, W., and Schoeppe, W., Immunological studies on membrane associated antigens from renal adenocarcinoma and human kidney cortex using poly- and monoclonal antibodies, *Protides Biol. Fluids*, 30, 333, 1983.
228. Luner, S. J., Ghose, T., and Cruz, H. N., Monoclonal antibodies to human renal tubular, glomerular and tumor associated antigens, *Fed. Proc.*, 44, 1436, 1985.
229. Vessella, R. L., Moon, T. D., Chiou, R.-K., Nowak, J. A., Arfman, E. W., Palme, D. F., Peterson, G. A., and Lange, P. H., Monoclonal antibodies to human renal cell carcinoma: recognition of shared and restricted tissue antigens, *Cancer Res.*, 45, 6131, 1985.

230. Andy, R. J., Finstad, C. L., Old, L. J., Lloyd, K. O., and Kornfled, R., The antigen identified by a mouse monoclonal antibody raised against human renal cancer cells is the adenosine deaminase binding protein, *J. Biol. Chem.*, 259, 12844, 1984.
231. Lindop, G. B. and Fleming, S., Renin in renal cell carcinoma — an immunocytochemical study using an antibody to pure human renin, *J. Clin. Pathol.*, 37, 27, 1984.
232. Concolino, G., De Silverio, F., Marocchi, A., and Bracci, U., Renal cancer steroid receptors: biochemical basis for endocrine therapy, *Eur. Urol.*, 5, 319, 1979.
233. Bloom, E. T., Serological recognition of common antigens on human transitional cell carcinomas, *Fed. Proc.*, 35, 548, 1976.
234. Bubenik, J., Perlmann, P., Helmstein, K., and Mosberger, G., Cellular and humoral immune responses to human urinary bladder carcinomas, *Int. J. Cancer*, 5, 310, 1970.
235. Fradet, Y., Cordon-Cardo, C., Thomson, T., Daly, M. E., Whitmore, W. F., Jr., Lloyd, K. O., Melamed, M. R., and Old, L. J., Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 224, 1984.
236. Levi, M. M., Antigenicity of ovarian and cervical malignancies with a view toward possible immunodiagnosis, *Am. J. Obstet. Gynecol.*, 109, 689, 1971.
237. McCoy, J. P. and Haines, H. G., The antigenicity and immunology of human cervical squamous cell carcinoma: a review, *Am. J. Obstet. Gynecol.*, 140, 329, 1981.
238. Goldenberg, D. M., Garner, T. F., Pant, K. D., and van Nagell, J. R., Jr., Identification of beta-oncofetal 1 antigen in cervical squamous cancer and its demonstration in neoplastic and normal tissues, *Cancer Res.*, 38, 1246, 1978.
239. Om, A., Wright, B. A., and Ghose, T., Keratin and carcinoembryonic antigen (CEA) as immunohistochemical markers for primary and metastatic carcinomas, submitted.
240. Fritsche, H. A., Freedman, R. S., Liu, F., Acomb, L. D., and Collinsworth, W. L., A survey of tumor markers in patients with squamous cell carcinoma of the uterine cervix, *Gynecol. Oncol.*, 14, 230, 1982.
241. Royston, I. and Aurelian, L., Immunofluorescent detection of herpesvirus antigens in exfoliated cells from human cervical carcinoma, *Proc. Natl. Acad. Sci. U.S.A.*, 67, 204, 1970.
242. Pasca, A. S., Kummerlandar, L., Pejtsik, B. et al., Herpes simplex virus-specific antigens in exfoliated cervical cells from women with and without cervical anaplasia, *Cancer Res.*, 36, 2130, 1976.
243. Seeger, R. C., Danon, Y. L., Zeltzer, P. M., Maidman, J. E., and Rayner, S. A., Expression of fetal antigens by human neuroblastoma cells, in *Advances in Neuroblastoma Research*, Vol. 12, Evans, A. E., Ed., Raven Press, New York, 1980, 199.
244. Byrd, R. L., Jonak, Z. L., and Kennett, R. H., Monoclonal antibodies against human neuroblastomas and leukemias: detection of metastasis and of molecules involved in growth regulation hybrid, in *Cancer Diagnosis and Treatment*, Mitchell, M. S. and Oettgen, H. F., Eds., Raven Press, New York, 1982, 219.
245. Reynolds, C. P. and Smith, R. G., A sensitive immunoassay for human neuroblastoma cells, in hybridomas in cancer diagnosis and treatment, in *Progress in Cancer Research and Therapy*, Vol. 21, Mitchell, M. S. and Oettgen, H. F., Eds., Raven Press, New York, 1982, 235.
246. Seeger, R. C., Expression of human thy-1 by neuroblastoma, glioma, sarcoma, and teratoma cells, in *Hybridomas in Cancer Diagnosis and Treatment*, Vol. 21, Mitchell, M. S. and Oettgen, H. F., Eds., Raven Press, New York, 1982, 231.
247. Carrel, S., De Tribolet, N., and Mach, J. P., Human melanoma- and glioma-associated antigen(s) identified by monoclonal antibodies, *Methods Cancer Res.*, 20, 317, 1982.
248. Sugimoto, T., Tatsumi, E., Kemshead, J. T., Helson, L., Green, A. A., and Minowada, J., Determination of cell surface membrane antigens common to both human neuroblastoma and leukemia-lymphoma cell lines by a panel of 38 monoclonal antibodies, *JNCI*, 73, 51, 1984.
249. Wilkstrand, C. J. and Bigner, D. D., Use of monoclonal antibodies in neurobiology and neurooncology, in *Monoclonal Antibodies in Cancer*, Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N.J., 1985, chap. 16.
250. Saito, M., Yu, R. K., and Cheung, N.-K. V., Ganglioside GD2 specificity of monoclonal antibodies to human neuroblastoma cell, in *Biochemical and Biophysical Research Communications*, Vol. 127, Academic Press, New York, 1985, 1.
251. Nudelman, E., Hakomori, S., Kannagi, R., Levery, S., Yek, M. Y., Hellstrom, K. E., and Hellstrom, I., Characterization of a human melanoma-associated ganglioside antigen defined by a monoclonal antibody, *J. Biol. Chem.*, 57, 12752, 1982.
252. Kennett, R. H. and Gilbert, F., Hybrid myelomas producing antibodies against a human neuroblastoma antigen present on fetal brain, *Science*, 203, 1120, 1979.
253. Cheung, N.-K. V., Saarinen, U. M., Neely, J. E., Landmeier, B., Donovan, D., and Coccia, P. F., Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells, *Cancer Res.*, 45, 2642, 1985.

254. Treleavan, J. G., Gibson, F. M., Ugelstad, J., Rembau, A., Phillips, T., Caine, G. D., and Kemshead, J. T., Removal of neuroblastoma cells from bone marrow with monoclonal antibodies conjugated to magnetic microspheres, *Lancet*, 1, 70, 1984.
255. Goldman, A., Vivian, G., Gordon, I., Pritchard, J., and Kemshead, J., Immunolocalization of neuroblastoma using radiolabelled monoclonal antibody UJ13SA, *J. Pediat.*, 105, 252, 1984.
256. Hobbs, J., Melino, G., and Riches, P., Successful uses of tumour markers in man, *Protides Biol. Fluids*, 31, 255, 1983.
257. Wahlstrom, T., Linder, E., Saksela, E., and Westermark, B., Tumor-specific membrane antigens in established cell lines from gliomas, *Cancer*, 34, 274, 1974.
258. Coakham, H., Surface antigen(s) common to human astrocytoma cells, *Nature (London)*, 250, 328, 1974.
259. Coakham, H. B. and Lakshmi, M. S., Tumour-associated surface antigen(s) in human astrocytomas, *Oncology*, 31, 233, 1975.
260. Miyake, E. and Kitamura, K., An attempt to detect cell surface antigens in cultured human brain tumors by mixed hemadsorption test, *Acta Neuropathol.*, 37, 27, 1971.
261. Brown, J. M. and Rosenberg, S. A., Serologic analysis of human solid tumor antigens, in *Immunological Approaches to Cancer Therapeutics*, Mihich, E., Ed., John Wiley & Sons, New York, 1982, 1.
262. Sikora, K., Alderson, T., Phillips, J., and Watson, J. V., Human hybridomas from malignant gliomas, *Lancet*, 1, 11, 1982.
263. Sikora, K. and Phillips, J., Human monoclonal antibodies to glioma cells, *Br. J. Cancer*, 43, 104, 1981.
264. Ghose, T., Ramakrishnan, S., Kulkarni, P., Blair, A. H., Vaughan, K., Nolido, H., Norvell, S. T., and Belitsky, P., Use of antibodies against tumor-associated antigens for cancer diagnosis and treatment, *Transplant. Proc.*, 13, 1970, 1981.
265. Pierce, G. B., Teratocarcinoma: model for a development concept of cancer, in *Current Topics of Developmental Biology*, Monroy, A. and Moscona, A. A., Eds., Academic Press, New York, 1967, 223.
266. Blaineau, C., Connan, F., Arnaud, D., Andrews, P., Williams, L., McIlhinney, J., and Avner, P., Definition of three species-specific monoclonal antibodies recognizing antigenic structures present on human embryonal carcinoma cells which undergo modulation during in vitro differentiation, *Int. J. Cancer*, 34, 487, 1984.
267. Williams, L. K., Sullivan, A., McIlhinney, R. A. J., and Neville, A. M., A monoclonal antibody marker of human primitive endoderm, *Int. J. Cancer*, 30, 731, 1982.
268. Andrews, P. W., Banting, G., Damjanov, I., Arnaud, D., and Avner, P., Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells, *Hybridoma*, 3, 347, 1984.
269. Rettig, M. J., Gordon-Cardo, C., Ng, J. S. C., Oettgen, H. F., Old, L. J., and Lloyd, K. O., High molecular-weight glycoproteins of human teratocarcinoma defined by monoclonal antibodies to carbohydrate determinants, *Cancer Res.*, 45, 815, 1985.
270. Shevinsky, L. H., Knowles, B. B., Damjanov, I., and Solter, D., Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells, *Cell*, 30, 697, 1982.
271. Fox, N., Damjamou, T., Knowles, B. B., and Solter, D., Stage-specific embryonic antigen 3 as a marker of visceral extraembryonic endoderm, *Dev. Biol.*, 103, 263, 1983.
272. Kannagi, R., Cochran, N. A., Ishigami, A. et al., Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells, *Embo. J.*, 2, 2355, 1983.
273. Kannagi, R., Levery, S. B., Ishigami, F., Hakomori, S., Shevinsky, L. H., Knowles, B. B., and Solter, D., New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stag-specific embryonic antigen 3, *J. Biol. Chem.*, 258, 8934, 1983.
274. Ballou, B., Levine, G., Hakala, T. R., and Solter, D., Tumor location detected with radioactively labeled monoclonal antibody and external scintography, *Science*, 206, 844, 1979.
275. Ballou, B., Reiland, J., Levine, G., Knowles, B., and Hakala, T. R., Tumor localization using $F(ab')_2$ mu from a monoclonal IGM antibody: pharmacokinetics, *J. Nucl. Med.*, 26, 283, 1985.
276. Ghose, T., Norvell, S. T., Guclu, A., Bodurtha, A., Tai, J., and MacDonald, A. S., Immunochemotherapy of malignant melanoma with chlorambucil-bound antimelanoma globulins: preliminary results in patients with disseminated disease, *JNCI*, 158, 845, 1977.
277. Goding, J. W., Antibody production by hybridomas, *J. Immunol. Methods*, 29, 285, 1980.
278. Galfre, G. and Milstein, C., Preparation of monoclonal antibodies: strategies and procedures, *Methods Enzymol.*, 73, 3, 1981.

279. Lennox, E. S., Monoclonal antibodies and tumor antigens — a perspective, in *Hybridoma Cancer Diagnosis Treatment*, Mitchell, M. S. and Oettgen, H. F., Eds., Raven Press, New York, 1982, 5.
280. Egan, M. L. and Henson, D. E., Monoclonal antibodies and breast cancer, *JNCI*, 68, 338, 1982.
281. Kahan, B. D., Pellis, N. R., LeGrue, S. J., and Tanaka, T., Immunotherapeutic effects of tumor-specific transplantation antigens released by 1-butanol, *Cancer*, 49, 1168, 1982.
282. Olsson, L., Human monoclonal antibodies in experimental cancer research, *JNCI*, 75, 397, 1985.
283. Sikora, K. and Wright, R., Human monoclonal antibodies to lung-cancer antigens, *Br. J. Cancer*, 43, 696, 1981.
284. Lowe, D. H., Handley, H. H., Schmidt, J., Royston, I., and Glassy, M. C., A human monoclonal antibody reactive with human prostate, *J. Urol.*, 132, 780, 1984.
285. Olsson, L., Kronstrom, H., Cambon-De Mouzon, A., Honsik, C., Brokin, T., and Jakobsen, B., Antibody producing human-human hybridomas. I. Technical aspects, *J. Immunol. Methods*, 61, 17, 1983.
286. Sikora, K., Alderson, T., Ellis, J., Phillips, J., and Watson, J., Human hybridomas from patients with malignant disease, *Br. J. Cancer*, 47, 135, 1983.
287. Sikora, K., Human monoclonal antibodies, *Br. Med. Bull.*, 40, 209, 1984.
288. Lamoyi, E. and Nisonoff, A., Preparation of $F(ab)_2$ fragments from mouse IgG of various subclasses, *J. Immunol. Methods*, 56, 235, 1983.
289. Kennel, S. J., Foote, L. J., Lankford, P. K., Johnson, M., Mitchell, T., and Braslawsky, G. R., Direct binding of radioiodinated monoclonal antibody to tumor cells: significance of antibody purity and affinity for drug targeting or tumor imaging, *Hybridoma*, 2, 297, 1983.
290. Mason, D. W. and Williams, A. F., The kinetics of antibody binding to membrane antigens in solution and at the cell surface, *Biochem. J.*, 187, 1, 1980.
291. Ghose, T., Tai, J., Norvell, S. T., Guclu, A., and MacDonald, A. S., Use of antibodies as carriers of radionuclides and cytotoxic drugs in the treatment and diagnosis of cancer, *Ann. N.Y. Acad. Sci.*, 227, 671, 1976.
292. Ghose, T., Guclu, A., Raman, R. R., and Blair, A. H., Inhibition of a mouse hepatoma by the alkylating agent Trenimon linked to immunoglobulins, *Cancer Immunol. Immunother.*, 13, 185, 1982.
293. Kennel, S. J., Binding of monoclonal antibody to protein antigen in fluid phase or bound to solid supports, *J. Immunol. Methods*, 55, 1, 1982.
294. Stuhlmiller, G. M., Sullivan, D. C., Vervaert, C. E., Croker, B. R., Harris, C. C., and Seigler, H. F., In vivo tumor localization using tumor-specific monkey xenoantibody, alloantibody, and murine monoclonal xenoantibody, *Ann. Surg.*, 194, 592, 1981.
295. Uadia, P., Blair, A. H., Ghose, T., and Ferrone, S., Uptake of methotrexate linked to polyclonal and monoclonal antimelanoma by a human melanoma cell line, *JNCI*, 74, 29, 1985.
296. Bale, W. F., Countreras, M. A., and Grady, E. D., Factors influencing localization of labeled antibodies in tumors, *Cancer Res.*, 40, 2960, 1980.
297. Guclu, A., Tai, J., and Ghose, T., Endocytosis of chlorambucil-bound anti-tumor globulin following "capping" in EL4 lymphoma cells, *Immunol. Commun.*, 4, 229, 1975.
298. Hakomori, S., Tumor associated carbohydrates antigens, *Annu. Rev. Immunol.*, 2, 103, 1984.
299. Kulkarni, P. N., Blair, A. H., Ghose, T., and Mammen, M., Conjugation of methotrexate to IgG antibodies and their $F(ab)_2$ fragments and the effect of conjugated methotrexate on tumor growth in vivo, *Cancer Immunol. Immunother.*, 19, 211, 1985.
300. Pressman, D., The development and use of radiolabeled antitumor antibodies, *Cancer Res.*, 40, 2960, 1980.
301. Bernstein, I. D. and Nowinski, R. C., Monoclonal antibody treatment of transplanted and spontaneous murine leukemia, in *Hybridomas in the Diagnosis and Treatment of Cancer*, Oettgen, H. and Mitchell, M., Eds., Raven Press, New York, 1982, 97.
302. Ghose, T., Blair, A. H., Uadia, P., Kulkarni, P. N., Goundalkar, A., Mezei, M., and Ferrone, S., Antibodies as carriers of cancer chemotherapeutic agents, *Ann. N.Y. Acad. Sci.*, 446, 213, 1985.
303. Houston, L. L., Nowinski, R. C., and Bernstein, I. D., Specific in vivo localization of monoclonal antibodies directed against the thy 1.1 antigen, *J. Immunol.*, 125, 837, 1980.
304. Herlyn, D. M., Steplewski, Z., Herlyn, M. F., and Koprowski, H., Inhibition of growth of colorectal carcinoma in nude mice by monoclonal antibody, *Cancer Res.*, 40, 717, 1980.
305. Moshakis, V., McIlhinney, R. A., Raghavan, D., and Neville, A. M., Localization of human tumour xenografts after i.v. administration of radiolabelled monoclonal antibodies, *Br. J. Cancer*, 44, 91, 1981.
306. Hellstrom, K. E., Doolittle, R. F., and Dreyer, W. J., Human melanoma-associated antigen P97 is structurally and functionally related to transferrin, *Nature (London)*, 295, 171, 1982.
307. Uadia, P., Blair, A. H., and Ghose, T., Tumor and tissue distribution of a methotrexate-anti-EL4 immunoglobulin conjugate in EL4 lymphoma-bearing mice, *Cancer Res.*, 44, 4263, 1984.

308. Scheinberg, D. A. and Strand, M., Leukemic cell targeting and therapy by monoclonal antibody, *Cancer Res.*, 42, 44, 1982.
309. Scheinberg, D. A. and Strand, M., Kinetic and catabolic considerations of monoclonal antibody targeting in erythroleukemic mice, *Cancer Res.*, 43, 265, 1983.
310. Badger, C. C., Krohn, K. A., Peterson, A. V., Shulman, H., and Bernstein, I. D., Experimental radiotherapy of murine lymphoma with 131-I-labelled anti-Thy 1.1 monoclonal antibody, *Cancer Res.*, 45, 1536, 1985.
311. Mann, B. D., Cohen, M. B., Saxton, R. E., Morton, D. L., Benedict, W. F., Korn, E. L., Spolter, L., Graham, L. S., Chang, C. C., and Burk, M. W., Imaging of human tumor xenografts in nude mice with radiolabeled monoclonal antibodies, *Cancer*, 54, 1318, 1984.
312. Miller, R. A., Maloney, D. G., Warnke, R., and Levy, R., Treatment of B cell lymphoma with monoclonal anti-idiotypic antibody, *N. Engl. J. Med.*, 306, 517, 1982.
313. Nadler, L. M., Stashenko, P., Hardy, D. W., Antman, K. H., and Schlossman, S. T., Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen, *Cancer Res.*, 40, 3147, 1980.
314. Dillman, R. O., Sobol, R. E., Collins, H. et al., T101 monoclonal antibody therapy in chronic lymphocytic leukemia, in *Hybridomas in Cancer Diagnosis and Treatment*, Oettgen, H. and Mitchell, M., Eds., Raven Press, New York, 1982, 151.
315. Godal, A., Fodstad, O., and Pihl, A., Antibody formation against the cytotoxic proteins abrin and ricin in humans and mice, *Int. J. Cancer*, 32, 515, 1983.
316. Ritz, J., Pesando, J. M., Sallan, S. E., Clavell, L. A., Notis-McConathy, J., Rosenthal, P., and Schlossman, S. F., Serotherapy of acute lymphoblastic leukemia with monoclonal antibody, *Blood*, 58, 141, 1981.
317. Hellstrom, E. K., Hellstrom, I., and Brown, J. P., Human tumor-associated antigens identified by monoclonal antibodies, *Springer Semin. Immunopathol.*, 5, 127, 1982.
318. Foulds, L., The experimental study of tumor progression: a review, *Cancer Res.*, 14, 327, 1954.
319. Olsson, L., Sorenson, H. R., and Behnke, O., Intratumoral phenotypic diversity of cloned human lung tumor cell lines and consequences for analyses with monoclonal antibodies, *Cancer*, 54, 1757, 1984.
320. Natali, P., Bigotti, A., Cavaliere, R., Liao, S.-K., Taniguchi, M., Matsui, M., and Ferrone, S., Heterogeneous expression of melanoma-associated antigens and HLA antigens by primary and multiple metastatic lesions removed from patients with melanoma, *Cancer Res.*, 45, 2883, 1985.
321. Burchiel, S. W., Martin, J. C., Imai, K., Ferrone, S., and Warner, N. L., Heterogeneity of HLA-A,B,Ia-like, and melanoma-associated antigen expression by human melanoma cell lines analysed with monoclonal antibodies and flow cytometry, *Cancer Res.*, 42, 4110, 1982.
322. Nadler, L. M., Stashenko, P., Hardy, R., and Schlossman, S. F., A monoclonal antibody defining lymphoma-associated antigen in man, *J. Immunol.*, 125, 570, 1980.
323. Tsuji, Y., Suzuki, T., Nishiura, H., Takemura, T., and Isojima, S., Identification of two different surface epitopes of human ovarian epithelial carcinomas by monoclonal antibodies, *Cancer Res.*, 45, 2358, 1985.
324. Mulshine, J. L., Cuttitta, F., and Minna, J. D., Lung cancer markers as detected by monoclonal antibodies, in *Monoclonal Antibodies in Cancer*, Sell, S. and Reisfeld, R. A., Eds., Humana Press, Clifton, N.J., 1985, 229.
325. Levy, R. and Miller, R. A., Biological and clinical implications of lymphocyte hybridomas: tumor therapy with monoclonal antibodies, *Ann. Res. Med.*, 34, 107, 1983.
326. Miller, R. A. and Levy, R., Response of cutaneous T cell lymphoma to therapy with hybridoma monoclonal antibody, *Lancet*, 2, 226, 1981.
327. Miller, R. A., Maloney, D. G., McKillop, J., and Levy, R., In vivo effects of murine hybridoma monoclonal antibody in a patient with T cell leukemia, *Blood*, 58, 78, 1981.
328. Miller, R. A., Oseroff, A. R., Stratte, P. T., and Levy, R., Monoclonal antibody therapeutic trials in seven patients with T cell lymphoma, *Blood*, 62, 988, 1983.
329. Dillman, R. O., Shawler, D. L., Dillman, J. B., Clutter, M., Wormsley, S. B., Markham, M., and Frisman, D., Monoclonal antibody therapy of cutaneous T cell lymphoma (CTCL), *Blood*, 62(Suppl. 1), 212, 1983.
330. Sears, H. F., Herlyn, D., Steplewski, Z., and Koprowski, H., Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma, *J. Biol. Response Modifiers*, 3, 138, 1984.
331. Foon, K. A., Schroff, R. W., Bunn, P. A., Mayer, D., Abrams, P. G., Fer, M., Ochs, J., Bottino, G. C., Sherwin, S. A., Carlo, D. J., and Herberman, E., Effects of monoclonal antibody therapy in patients with chronic lymphocytic leukemia, *Blood*, 64, 1085, 1984.

332. Kishida, K., Masuho, Y., Saito, M., Hara, T., and Fuji, H., Ricin A-chain conjugated with monoclonal anti-L1210 antibody: in vitro and in vivo antitumor activity, *Cancer Immunol. Immunother.*, 16, 93, 1983.
333. Shen, W.-C. and Ryser, J.-P., cis-Aconityl spacer between daunomycin and macromolecular carriers: a model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate, *Biochem. Biophys. Res. Commun.*, 102, 1048, 1981.
334. Gallego, J., Price, M. R., and Baldwin, R. W., Preparation of four daunomycin-monoclonal antibody 191T/36 conjugates with anti-tumor activity, *Int. J. Cancer*, 33, 737, 1984.
335. Zunino, F., Gambetta, R., Vigevani, A., Penco, S., Geroni, C., and Di Marco, A., Biologic activity of daunorubicin linked to proteins via the methylketone side chain, *Tumori*, 67, 521, 1981.
336. Kato, Y., Tsukada, Y., Hara, T., and Hirai, H., Enhanced antitumor activity of mitomycin C conjugated with anti-alpha-fetoprotein antibody by a novel method of conjugation, *J. Appl. Biochem.*, 5, 313, 1983.
337. de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F., Lysosomotropic agents, *Biochem. Pharmacol.*, 23, 2495, 1974.
338. Monsigny, M., Kieda, C., Roche, A.-C., and Delmotte, F., Preparation and biological properties of a covalent antitumor drug-arm-carrier (DAC conjugate), *FEBS Lett.*, 119, 181, 1980.
339. Trouet, A., Masquelier, M., Baurain, R., and Deprez de Campeneere, D., A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for lysosomotropic drug-carrier conjugate, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 626, 1982.
340. Edwards, D. C., Targeting potential of antibody conjugates, *Pharmacol. Ther.*, 23, 147, 1983.
341. Heney, G. and Orr, G. A., The purification of avidin and its derivatives on 2-iminobiotin-6-aminohexyl-Sepharose 4B, *Anal. Biochem.*, 114, 92, 1981.
342. Hashimoto, N., Takatsu, K., Masuho, Y., Kishida, K., Hara, T., and Hamaoka, T., Selective elimination of a B cell subset having acceptor site(s) for T cell-replacing factor (TRF) with biotinylated antibody to the acceptor site(s) and avidin-ricin A-chain conjugate, *J. Immunol.*, 132, 129, 1984.
343. Shen, W.-C. and Ryser, H. J.-P., Selective killing of Fc-receptor-bearing tumor cells through endocytosis of a drug-carrying immune complex, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 1445, 1984.
344. Raso, V., Antibody mediated delivery of toxic molecules to antigen bearing target cells, *Immunol. Rev.*, 62, 93, 1982.
345. Kulkarni, P. N., Blair, A. H., and Ghose, T., Covalent binding of methotrexate to immunoglobulins and the effect of antibody-linked drug on tumor growth in vivo, *Cancer Res.*, 41, 2700, 1981.
346. Lutter, L. C., Ortlander, F., and Fasold, H., The use of a new series of cleavable protein crosslinkers on the *Escherichia coli* ribosome, *FEBS Lett.*, 48, 288, 1974.
347. Hurwitz, E., Wilchek, M., and Pitha, J., Soluble macromolecules as carriers for daunorubicin, *J. Appl. Biochem.*, 2, 25, 1980.
348. Trouet, A., Increased selectivity of drugs by linking to carriers, *Eur. J. Cancer*, 14, 105, 1978.
349. Lesur, B., Masquelier, M., Baurain, R., and Trouet, A., Covalent linkage of anthracyclines to macromolecular carriers, *J. Cell. Biochem.*, Suppl. 9A, 437, 1985.
350. Shen, W.-C. and Ryser, J.-P., Conjugation of methotrexate to 5 basic polypeptides: comparison of inhibitory effect on cell defective in drug transport, *Fed. Proc.*, 49, 642, 1981.
351. Garnett, M. C., Embleton, M. J., Jacobs, E., and Baldwin, R. W., Preparation and properties of a drug-carrier-antibody conjugate showing selective antibody-directed cytotoxicity in vitro, *Int. J. Cancer*, 31, 661, 1983.
352. Rowland, G. F., O'Neill, G. J., and Davies, D. A. L., Suppression of tumour growth in mice by a drug-antibody conjugate using a novel approach to linkage, *Nature (London)*, 255, 487, 1975.
353. Tsukada, Y., Kato, Y., Umemoto, N., Takeda, Y., Hara, T., and Hirai, H., An anti-alpha-fetoprotein antibody-daunomycin conjugate with a novel poly-L-glutamic acid derivative as intermediate carrier, *JNCI*, 73, 721, 1984.
354. Carlsson, J., Drevin, H., and Axen, R., Protein thiolation and reversible protein-protein conjugation: N-succinimidyl 3-(2-pyridyldithio) propionate, a new hetero-bifunctional reagent, *Biochem. J.*, 173, 723, 1978.
355. Kato, Y., Umemoto, N., Kayama, Y., Fukushima, H., Takeda, Y., Hara, T., and Tsukada, Y., A novel method of conjugation of daunomycin with antibody with a poly-L-glutamic acid derivative as intermediate drug carrier. An anti-alpha-fetoprotein antibody-daunomycin conjugate, *J. Med. Chem.*, 27, 1602, 1984a.
356. Kato, Y., Saito, M., Fukushima, H., Takeda, Y., and Hara, T., Antitumor activity of 1-B-D-arabinofuranosylcytosine conjugated with poly-L-glutamic acid and its derivatives, *Cancer Res.*, 44, 25, 1984.
357. Arnon, R. and Sela, M., In vitro and in vivo efficacy of conjugates of daunomycin with anti-tumor antibodies, *Immunol. Rev.*, 62, 5, 1982.

358. Manabe, Y., Tsubota, T., Haruta, Y., Okazaki, M., Haisa, S., Nakamura, K., and Kimura, I., Production of a monoclonal antibody-bleomycin conjugate utilizing dextran-T40 and the antigen-targeting cytotoxicity of the conjugate, *Biochem. Biophys. Res. Commun.*, 115, 1009, 1983.
359. Manabe, Y., Tsubota, T., Haruta, Y., Kataoka, K., Okazaki, M., Haisa, S., Nakamura, K., and Kimura, I., Production of a monoclonal antibody-mitomycin C conjugate utilizing dextran-T40, and its biological activity, *Biochem. Pharmacol.*, 34, 289, 1985.
360. Manabe, Y., Tsubota, T., Haruta, Y., Kataoka, K., Okazaki, M., Haisa, S., Nakamura, K., and Kimura, I., Production of a monoclonal antibody-methotrexate conjugate utilizing dextran-T40 and its biological activity, *J. Lab. Clin. Med.*, 104, 445, 1984.
361. Hurwitz, E., Kashi, R., Arnon, R., Wilchek, M., and Sela, M., The covalent linking of two nucleotide analogues to antibodies, *J. Med. Chem.*, 28, 137, 1985.
362. Moolton, F. L., Schreiber, B. M., and Zajdel, S. H., Antibodies conjugated to potent cytotoxins as specific antitumor agents, *Immunol. Rev.*, 62, 47, 1982.
363. Thorpe, P. E., Mason, D. W., Brown, A. N. F., Simmonds, J., Ross, W. C. J., Cumber, A. J., and Forrester, J. A., Selective killing of malignant cells in a leukaemic rat bone marrow using an antibody-ricin conjugate, *Nature (London)*, 297, 594, 1982.
364. Thorpe, P. E. and Ross, W. C. J., The preparation and cytotoxic properties of antibody-toxin conjugates, *Immunol. Rev.*, 62, 119, 1982.
365. Vitetta, E. S., Krolick, K. A., and Uhr, J. W., Neoplastic B cells as targets for antibody-ricin A chain immunotoxins, *Immunol. Rev.*, 62, 159, 1982.
366. Jansen, F. K., Blythman, H. E., Carriere, D., Casellas, P., Gros, O., Gros, P., Laurent, J. C., Paolucci, F., Pau, B., and Poncelet, P., Immunotoxins: hybrid molecules combining high specificity and potent cytotoxicity, *Immunol. Rev.*, 62, 185, 1982.
367. Cumber, A. J., Forrester, J. A., Foxwell, B. M. J., Ross, W. C. J., and Thorpe, P. E., Preparation of antibody-toxin conjugates, *Methods Enzymol.*, 112, 207, 1985.
368. Domingo, L. D. and Trowbridge, I. S., Transferrin receptor as target for antibody-drug conjugates, *Methods Enzymol.*, 112, 238, 1985.
369. Yoshitake, S., Yamada, Y., Ishikawa, E., and Masseyeff, R., Conjugation of glucose oxidase from *Aspergillus niger* and rabbit antibodies using N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide, *Eur. J. Biochem.*, 101, 395, 1979.
370. Masuho, Y., Hara, T., and Noguchi, T., Preparation of a hybrid of fragment Fab′ of antibody and fragment A of diphtheria toxin and its cytotoxicity, *Biochem. Biophys. Res. Commun.*, 90, 320, 1979.
371. Rector, E. S., Schwenk, R. J., Tse, K. S., and Sehon, A., A method for the preparation of protein-protein conjugates of predetermined composition, *J. Immunol. Methods*, 24, 321, 1978.
372. Thorpe, P. E., Ross, W. C. J., Brown, A. N. F., Meyers, C., Cumber, A. J., Foxwell, B. M. J., and Forrester, J. A., Blockade of the galactose binding sites of ricin by its linkage to antibody. Specific cytotoxic effects of the conjugates, *Eur. J. Biochem.*, 140, 63, 1984.
373. Uckun, F. M., Ramakrishnan, S., and Houston, L. L., Immunotoxin-mediated elimination of clonogenic tumor cells in the presence of human bone marrow, *J. Immunol.*, 134, 2010, 1985.
374. Vitetta, E. S., Cushley, W., and Uhr, J. W., Synergy of ricin A chain-containing immunotoxins and ricin B chain-containing immunotoxins in in vitro killing of neoplastic human B cells, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 6332, 1983.
375. Epstein, J., Rosenthal, R. W., and Ess, R. J., Use of gamma-(4-nitrobenzyl)-pyridine as analytical reagent for ethylenimines and alkylating agents, *Anal. Chem.*, 27, 1435, 1955.
376. Bernier, L. G., Gaudreault, R. C., Page, M., and Joly, L. P., Fluorescence determination of microconcentrations of chlorambucil after photoactivation, *J. Pharm. Sci.*, 73, 1157, 1984.
377. Pelham, H. R. B. and Jackson, R. J., An efficient mRNA-dependent translation system from reticulocyte lysate, *Eur. J. Biochem.*, 67, 247, 1976.
378. Leonard, J. E., Taetle, R., To, D., and Rhyner, K., Preclinical studies on the use of selective antibody-ricin conjugates in autologous bone marrow transplantation, *Blood*, 65, 1149, 1985.
379. Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., and Bunn, P. A., Jr., Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess, *J. Immunol. Methods*, 72, 77, 1984.
380. Gregoriadis, G., Liposomes as drug carriers, *Pharma Int.*, 4, 33, 1983.
381. Weinstein, J. N. and Leserman, L. D., Liposomes as drug carriers in cancer chemotherapy, *Pharmacol. Ther.*, 24, 207, 1984.
382. Barbet, J., Machy, P., and Leserman, L. D., Monoclonal antibody covalently coupled to liposomes: specific targeting to cells, *J. Supramolec. Struct. Cell Biochem.*, 16, 243, 1981.
383. Endoh, J., Suzuki, Y., and Hashimoto, Y., Antibody coating of liposomes with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and the effect on target specificity, *Immunol. Methods*, 44, 79, 1981.
384. Jansons, V. K. and Mallet, P. L., Targeted liposomes: a method for preparation and analysis, *Anal. Biochem.*, 111, 54, 1981.

385. Martin, F. J., Hubbell, W. L., and Papahadjopoulos, D., Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab′ fragments via disulfide bonds, *Biochemistry,* 20, 4229, 1981.

386. Shen, D., Huang, A., and Huang, L., An improved method for covalent attachment of antibody to liposomes, *Biochim. Biophys. Acta,* 689, 31, 1982.

387. Goldmacher, V. S., Immobilization of protein molecules on liposomes. Anchorage by artificially bound unsaturated hydrocarbon tails, *Biochem. Pharmacol.,* 32, 1207, 1983.

388. Heath, T. D., Macher, B. A., and Papahadjopoulos, D., Covalent attachment of immunoglobulins to liposomes via glycosphingolipids, *Biochim. Biophys. Acta,* 640, 66, 1981.

389. Goundalkar, A., Ghose, T., and Mezei, M., Covalent binding of antibodies to liposomes using a novel lipid derivative, *J. Pharm. Pharmacol.,* 36, 465, 1984.

390. Mayhew, E. and Papahadjopoulos, D., Therapeutic applications of liposomes, in *Liposomes,* Ostro, M. J., Ed., Marcel Dekker, New York, 1983, 289.

391. Matthay, K. K., Heath, T. D., and Papahadjopoulos, D., Specific enhancement of drug delivery to AKR lymphoma by antibody-targeted small unilamellar vesicles, *Cancer Res.,* 44, 1880, 1984.

392. Rowland, G. F., Effective antitumour conjugates of alkylation drug and antibody using dextran as the intermediate carrier, *Eur. J. Cancer,* 13, 593, 1977.

393. Brent, L., Horsburgh, T., Rowland, G., and Wood, P., Failure to increase the in vivo immunosuppressive activity of antilymphocyte globulin by conjugation with melphalan, *Transplantation,* 29, 280, 1980.

394. Ghose, T. and Nigam, S., Antibody as carrier of chlorambucil, *Cancer,* 29, 1398, 1972.

395. Ghose, T., Guclu, A., and Tai, J., Suppression of an AKR lymphoma by antibody and chlorambucil, *JNCI,* 55, 1353, 1975.

396. Flechner, I., The cure and concomitant immunization of mice bearing Ehrlich ascites tumours by treatment with an antibody-alkylating agent complex, *Eur. J. Cancer,* 9, 741, 1973.

397. Hirschberg, H., Rowland, G., and Thorsby, E., In vitro immunosuppressive effects of cytotoxic agents conjugated to antihuman lymphocyte globulin, *Transplantation,* 26, 292, 1978.

398. Tai, J., Blair, A. H., and Ghose, T., Tumor inhibition by chlorambucil covalently linked to antitumor globulin, *Eur. J. Cancer,* 15, 1357, 1979.

399. Latif, Z. A., Lozzio, B. B., Wust, C. J., Krauss, S., Aggio, M. C., and Lozzio, C. B., Evaluation of drug-antibody conjugates in the treatment of human myelosarcomas transplanted in nude mice, *Cancer,* 45, 1326, 1980.

400. De Weger, R. A., Dullens, H. F. J., Cumber, A. J., Smith, A., Thorpe, P. E., Brown, A., Williams, R. H., and Davies, A. J. S., Eradication of murine lymphoma and melanoma cells by chlorambucil-antibody complexes, *Immunol. Rev.,* 62, 29, 1982.

401. Tung, E., Goust, J. M., Chen, W. Y., Kang, S. S., Wang, I. Y., and Wang, A. C., Cytotoxic effect of anti-idiotype antibody-chlorambucil conjugates against human lymphoblastoid cells, *Immunology,* 50, 57, 1983.

402. Bernier, L. G., Page, M., Gaudreault, R. C., and Joly, L. P., A chlorambucil-anti-CEA conjugate cytotoxic for human colon adenocarcinoma cells in vitro, *Br. J. Cancer,* 49, 245, 1984.

403. Blakeslee, D. and Kennedy, J. C., Factors affecting the non-covalent binding of chlorambucil to rabbit immunoglobulin, *J. Cancer Res.,* 34, 882, 1974.

404. Blakeslee, D., Chen, M., and Kennedy, J. C., Aggregation of chlorambucil in vitro may cause misinterpretation of protein binding data, *Br. J. Cancer,* 31, 689, 1975.

405. Ross, W. C. J., The interaction of chlorambucil with human gamma-globulin, *Chem. Biol. Interactions,* 8, 261, 1974.

406. Ross, W. C. J., The conjugation of chlorambucil with human gamma-globulin, *Chem. Biol. Interactions,* 10, 169, 1975.

407. Jolivet, J., Cowan, K. H., Curt, G. A., Clendeninn, N. J., and Chabner, B. A., The pharmacology and clinical use of methotrexate, *N. Engl. J. Med.,* 309, 1094, 1983.

408. Uadia, P., Blair, A. H., and Ghose, T., Uptake of methotrexate linked to an anti-EL4-lymphoma antibody by EL4 cells, *Cancer Immunol. Immunother.,* 16, 127, 1983.

409. Imai, K., Nakanishi, T., Noguchi, T., Yachi, A., and Ferrone, S., Selective in vitro toxicity of purothionin conjugated to the monoclonal antibody 225.285 to a human molecular-weight melanoma-associated antigen, *Cancer Immunol. Immunother.,* 15, 206, 1983.

410. Embleton, M. J., Garnett, M. C., and Baldwin, R. W., Use of an anti-tumour monoclonal antibody for targeting MTX, *Protides Biol. Fluids,* 32, 429, 1984.

410a. Garnett, M. C. and Baldwin, R. W., An improved synthesis of a methotrexate-albumin-791T/36 monoclonal antibody conjugate cytotoxic to human osteogenic sarcoma cell lines, *Cancer Res.,* 46, 2407, 1986.

411. Tsukada, Y., Bischof, W. K.-D., Hibi, N., Hirai, H., Hurwitz, E., and Sela, M., Effect of a conjugate of daunomycin and antibodies to rat alpha-fetoprotein on the growth of alpha-fetoprotein-producing tumor cells, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 621, 1982.
412. Hurwitz, E., Levy, R., Maron, M., Wilchek, M., Arnon, R., and Sela, M., The covalent binding of daunomycin and adriamycin to antibodies with retention of both drug and antibody activities, *Cancer Res.*, 35, 1175, 1975.
413. Lee, F. H., Berczi, I., Fujimoto, S., and Sehon, A., The use of antifibrin antibodies for the destruction of tumor cells. III. Complete regression of MC-D sarcoma in guinea-pigs by conjugates of daunomycin with antifibrin antibodies, *Cancer Immunol. Immunother.*, 5, 201, 1978.
414. Lee, F. H., Fujimoto, S., and Sehon, A. H., The use of antifibrin antibodies for the destruction of tumor cells. Localization of antifibrin antibodies in a methylcholanthrene-induced sarcoma in guinea pigs, *Cancer Immunol. Immunother.*, 5, 187, 1978.
415. Belles-Isles, M. and Page, M., In vitro activity of daunomycin-anti-alphafoetoprotein conjugates on mouse hepatoma cells, *Br. J. Cancer*, 41, 841, 1980.
416. Belles-Isles, M. and Page, M., Anti-oncofoetal proteins for targeting cytotoxic drugs, *Int. J. Immunopharmacol.*, 3, 97, 1981.
417. Page, M., Delorme, F., Lafontaine, F., and Dumas, L., Chemotherapy with daunorubicin-anti-CEA conjugates in human colon adenocarcinoma grafted in nude mice, *Semin. Oncol.*, 11, 56, 1984.
418. Buchegger, F., Haskell, C. M., Schreyer, M. et al., Radiolabeled fragments of monoclonal antibodies against carcinoembryonic antigen for localization of human colon carcinoma grafted in nude mice, *J. Exp. Med.*, 158, 413, 1983.
419. Goldenberg, D. M., Preston, D. F., Primus, F. J., and Hansen, H. J., Photoscan localization of GW-39 tumors in hamsters using radiolabeled anti-carcinoembryonic antigen immunoglobulin G, *Cancer Res.*, 34, 1, 1974.
420. Mach, J. P., Carrel, S., Merenda, C., Sordat, B., and Cerottini, J. C., In vivo localization of radiolabeled antibodies to carcinoembryonic antigen in human colon carcinoma grafted into nude mice, *Nature (London)*, 248, 704, 1974.
421. Embleton, M. J., Garnett, M. C., Jacobs, E., and Baldwin, R. W., Antigenicity and drug susceptibility of human osteogenic sarcoma cells "escaping" a cytotoxic methotrexate-albumin-monoclonal antibody conjugate, *Br. J. Cancer*, 49, 559, 1984.
422. Shen, W.-C. and Ryser, J.-P., Poly(L-lysine) and poly(D-lysine) conjugates of methotrexate: different inhibitory effect on drug resistant cells, *Mol. Pharmacol.*, 16, 614, 1979.
423. Ryser, J.-P. and Shen, W.-C., Conjugation of methotrexate to poly(L-lysine) as a potential way to overcome drug resistance, *Chem. Biol. Interactions*, 45, 1207, 1980.
424. Bernstein, A., Hurwitz, E., Maron, R., Arnon, R., Sela, M., and Wilchek, M., Higher antitumor efficacy of daunomycin when linked to dextran: in vivo and in vitro studies, *JNCI*, 60, 379, 1978.
425. Hurwitz, E., Maron, M., Bernstein, A., Wilchek, M., Sela, M., and Sela, M., The effect in vivo of chemotherapeutic drug-antibody conjugates in two murine experimental tumor systems, *Int. J. Cancer*, 21, 747, 1978.
426. Hurwitz, E., Kashi, R., Burowsky, D., Arnon, R., and Haimovich, J., Site-directed chemotherapy with a drug bound to anti-idiotypic antibody to a lymphoma cell-surface IgM, *Int. J. Cancer*, 31, 745, 1983.
427. Pimm, M. V., Jones, J. A., Price, M. R., Middle, J. G., Embleton, M. J., and Baldwin, R. W., Tumour localization of monoclonal antibodies against a rat mammary carcinoma and suppression of tumour growth with adriamycin-antibody conjugates, *Cancer Immunol. Immunother.*, 12, 125, 1982.
428. Rowland, G. F., Simmonds, R. G., Corvalan, J. R. F., Marsden, C. H., Johnson, J. R., Woodhouse, C. S., Ford, C. H. J., and Newman, C. E., The potential use of monoclonal antibodies in drug targeting, *Protides Biol. Fluids*, 29, 921, 1981.
429. Rowland, G. F., Axton, C. A., Baldwin, R. W., Brown, J. P., Corvalan, J. R. F., Embleton, M. J., Gore, V. A., Hellstrom, I., Hellstrom, K. E., Jacobs, E., Marsden, C. H., Pimm, M. V., Simmonds, R. G., and Smith, W., Antitumor properties of vindesine-monoclonal antibody conjugates, *Cancer Immunol. Immunother.*, 19, 1, 1985.
430. Johnson, J. R., Ford, C. H. J., Newman, C. E., Woodhouse, C. S., Rowland, G. F., and Simmonds, R. G., A vindesine-anti-CEA conjugate cytotoxic for human cancer cells in vitro, *Br. J. Cancer*, 44, 372, 1981.
431. Embleton, M. J., Rowland, G. F., Simmonds, R. G., Jacobs, E., Marsden, C. H., and Baldwin, R. W., Selective cytotoxicity against human tumor cells by a vindesine-monoclonal antibody conjugate, *Br. J. Cancer*, 47, 43, 1983.
432. Ford, C. H. J., Newman, C. E., Johnson, J. R., Woodhouse, C. S., Reeder, T. A., Rowland, G. F., and Simmonds, R. G., Localisation and toxicity study of a vindesine-anti-CEA conjugate in patients with advanced cancer, *Br. J. Cancer*, 47, 35, 1983.

433. Conrad, R. A., Cullinan, G. J., Gerzon, K., and Poore, G. A., Structure activity relationships of dimeric catharanthus alkaloid. II. Experimental anti-tumour activities of N-substituted desacetyl vinblastine amide (vindesine) sulphates, *J. Med. Chem.*, 22, 391, 1979.
434. Barnett, C. J., Cullinan, G. J., Gerzon, K., Hoying, R. C., Sweeney, M. J., and Todd, G. C., Structure-activity relationships of dimeric catharanthus alkoloids. I. Deacetylvinblastine amide (vindesine) sulfate, *J. Med. Chem.*, 21, 88, 1978.
435. Hurwitz, E., Kashi, R., Arnon, R., Wilchek, M., and Sela, M., The covalent linking of two nucleotide analogues to antibodies, *J. Med. Chem.*, 28, 137, 1985.
436. Suzuki, T., Sato, E., Goto, K., Katsurada, Y., Unno, K., and Takahashi, T., The preparation of mitomyein C, adriamycin and daunomycin covalently bound to antibodies as improved cancer chemotherapeutic agents, *Chem. Pharm. Bull.*, 29, 844, 1981.
437. Kimura, I., Ohnoshi, T., Tsubota, T., Kobayashi, T., and Abe, S., Production of tumor antibody-neocarzinostatin (NCS) conjugate and its biological activity, *Cancer Immunol. Immunother.*, 7, 235, 1980.
438. Kimura, I., Tsubota, T., Ohnoshi, T., Sato, Y., Okazaki, M., Manabe, Y., and Abe, S., In vivo antitumor activity of neocarzinostatin (NCS)-tumor antibody conjugate against a transplantable human leukemia cell line (BALL-1), *Jpn. J. Clin. Oncol.*, 13, 425, 1983.
438a. Luders, G., Kohnlein, W., Sorg, C., and Bruggen, J., Selective toxicity of neocarzinostatin-monoclonal antibody conjugates to the antigen-bearing human melanoma cell line in vitro, *Cancer Immunol. Immunother.*, 20, 85, 1985.
439. Muirhead, M., Martin, P. J., Torok-Storb, B., Uhr, J. W., and Vitetta, E., Use of an antibody-ricin A-chain conjugate to delete neoplastic B cells from human bone marrow, *Blood*, 62, 327, 1983.
440. Poncelet, P., Blythman, H. E., Carriere, D., Casellas, P., Dussossoy, D., Gros, O., Gros, P., Jansen, F. K., Laurent, J. C., Liance, M. C., Vidal, H., and Voisin, G. A., Present potential of immunotoxins, *Behring Inst. Mitt.*, No. 74, 94, 1984.
441. Thorpe, P. E., Edwards, D. C., Davies, A. J. S., and Ross, W. C. J., Monoclonal antibody-toxin conjugates: aiming the magic bullet, in *Monoclonal Antibodies in Clinical Medicine*, McMichael, A. J. and Fabre, J. W., Eds., Academic Press, London, 1982, 167.
442. Uhr, J. W., Immunotoxins: harnessing nature's poisons, *J. Immunol.*, 133, 1, 1984.
443. Hofstaetter, T., Gronski, P., and Seiler, F. R., Immunotoxins — theoretical and practical aspects, *Behring Inst. Mitt.*, No. 74, 113, 1984.
444. Yamaizumi, M., Mekada, E., Uchida, T., and Okada, Y., One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell, *Cell*, 15, 145, 1978.
445. Eiklid, K., Olsnes, S., and Pihl, A., Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells, *Exp. Cell Res.*, 126, 321, 1980.
446. Neville, D. M. and Chang, T. M., Receptor-mediated protein transport into cells. Entry mechanisms for toxins, hormones, antibodies, viruses, lysosomal hydrolases, asialoglycoproteins, and carrier proteins, *Curr. Top. Membr. Transp.*, 10, 66, 1978.
447. Youle, R. J., Murray, G. J., and Neville, D. M., Studies on the galactose-binding site of ricin and the hybrid toxin man6P-ricin, *Cell*, 23, 551, 1981.
448. Youle, R. J. and Neville, D. M., Kinetics of protein synthesis inactivation by ricin-anti-thy 1.1 monoclonal antibody hybrids, *J. Biol. Chem.*, 257, 1598, 1982.
449. Edwards, D. C., Ross, W. C. J., Cumber, A. J., McIntosh, D., Smith, A., Thorpe, P. E., Brown, A., Williams, R. H., and Davies, A. J. S., A comparison of the in vitro and in vivo activities of conjugates of anti-mouse lymphocyte globulin and abrin, *Biochim. Biophys. Acta*, 717, 272, 1982.
450. Ross, W. C. J., Thorpe, P. E., Cumber, A. J., Edwards, D. C., Hinson, C. A., and Davies, A. J. S., Increased toxicity of diphtheria toxin for human lymphoblastoid cells following covalent linkage to anti-(human lymphocyte) globulin, *Eur. J. Biochem.*, 104, 381, 1980.
451. Vitetta, E. S., Fulton, R. J., and Uhr, J. W., Cytotoxicity of a cell-reactive immunotoxin containing ricin A chain is potentiated by an anti-immunotoxin containing ricin B chain, *J. Exp. Med.*, 160, 341, 1984.
451a. Thorpe, P. E., Brown, A. N. F., Bremner, J. A. G., Jr., Foxwell, B. M. J., and Stirpe, F., An immunotoxin composed of monoclonal anti-thy 1.1 antibody and a ribosome inactivating protein from *Saponaria officinalis:* potent antitumor effects in vitro and in vivo, *JNCI*, 75, 151, 1985.
452. Irvin, J. D., Pokeweed antiviral protein, *Pharmacol. Ther.*, 21, 371, 1983.
453. Uckun, F. M., Ramakrishnan, S., and Houston, L. L., Ex vivo elimination of neoplastic cells from human marrow using an anti-Mr 41,000 protein immunotoxin: potentiation by ASTA Z7557, *Blut*, 50, 19, 1985.
454. Richards, F. M. and Vithayathil, P. J., The preparation of subtilisin-modified ribonuclease and the separation of the peptide and protein components, *J. Biol. Chem.*, 234, 1495, 1969.

455. Raso, V., Ritz, J., Basala, M., and Schlossman, S. F., Monoclonal antibody-ricin A chain conjugate selectively cytotoxic for cells bearing the common acute lymphocytic leukemia antigen, *Cancer Res.*, 42, 457, 1982.
456. Chang, T.-M. and Neville, D. M., Jr., Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulfide conjugate. I. Synthesis of diphtheria toxin fragment A-S-S-human placental lactogen with methyl-5-bromovalerimidate, *J. Biol. Chem.*, 252, 1501, 1977.
457. Bernhard, M. I., Foon, K. A., Oeltmann, T. N., Key, M. E., Hwang, K. M., Clarke, G. C., Christensen, W. L., Hoyer, L. C., Hanna, M. G., and Oldham, R. K., Guinea pig line 10 hepatocarcinoma model: characterization of monoclonal antibody and in vivo effect of unconjugated antibody and antibody conjugated to diphtheria toxin A chain, *Cancer Res.*, 43, 4420, 1983.
458. Key, M. E., Bernhard, M. I., Hoyer, L., Foon, K. A., Oldham, R. K., and Hanna, M. G., Guinea pig line 10 hepatocarcinoma model for monoclonal antibody serotherapy: in vivo localization of a monoclonal antibody in normal and malignant tissues, *J. Immunol.*, 130, 1451, 1983.
459. Krolick, K. A., Uhr, J. W., and Vitetta, E. S., Selective killing of leukemia cells by antibody-toxin conjugates: implications for autologous bone marrow transplantation, *Nature (London)*, 295, 604, 1982.
460. Akiyama, S.-I., Seth, P., Pirker, R., FitzGerald, D., Gottesman, M. M., and Pastan, I., Potentiation of cytotoxic activity of immunotoxins on cultured human cells, *Cancer Res.*, 45, 1005, 1985.
461. FitzGerald, D. J. P., Trowbridge, I. S., Pastan, I., and Willingham, M. C., Enhancement of toxicity of anti-transferrin receptor antibody-pseudomonas exotoxin conjugates by adenovirus, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 4134, 1983.
462. Akiyama, S.-I., Gottesman, M. M., Hanover, J. A., FitzGerald, D. J. P., Willingham, M. C., and Pastan, I., Verapamil enhances the toxicity of conjugates of epidermal growth factor with *Pseudomonas* exotoxin and antitransferrin receptor with *Pseudomonas* exotoxin, *J. Cell. Physiol.*, 120, 271, 1984.
463. FitzGerald, D. J. P., Padmanabhan, R., Pastan, I., and Willingham, M. C., Adenovirun-induced release of epidermal growth factor and pseudomonas toxin into the cytosolof KB cells during receptor-mediated endocytosis, *Cell*, 32, 606, 1983.
464. Terouanne, B., Nicolas, J. C., Descomps, B., and De Paulet, A., Coupling of delta-5,3-ketosteroid isomerase to human placental lactogen with intermolecular disulfide bond formation. Use of this conjugate for a sensitive enzyme immunoassay, *J. Immunol. Methods*, 35, 267, 1980.
465. Trowbridge, I. S. and Domingo, D. L., Anti-transferrin receptor monoclonal antibody and toxin antibody conjugates affect growth of human tumor cells, *Nature (London)*, 294, 171, 1981.
466. Neville, D. M., Jr. and Youle, R. J., Monoclonal antibody-ricin or ricin A chain hybrids: kinetic analysis of cell killing for tumor therapy, *Immunol. Rev.*, 62, 75, 1982.
467. McIntosh, D. P., Edwards, D. C., Cumber, A. J., Parnell, G. D., Dean, C. J., and Ross, W. C. J., Ricin B chain converts a non-cytotoxic antibody-ricin A chain conjugate, *FEBS Lett.*, 164, 17, 1983.
468. Eckelman, W. C., Paik, C. H., and Reba, R. C., Radiolabeling of antibodies, *Cancer Res.*, 40, 3036, 1980.
469. Ghose, T. and Guclu, A., Cure of mouse lymphoma with radio-iodinated antibody, *Eur. J. Cancer*, 10, 787, 1974.
470. Order, S. E., The use of radiolabeled antibody in cancer chemotherapy, *Dev. Oncol.*, 26, 257, 1984.
471. Ettinger, D. S., Order, S. E., Wharam, M. D., Parker, M. K., Klein, J. L., and Leichner, P. D., Phase I-II study of isotopic immunoglobulin therapy for primary liver cancer, *Cancer Treat. Rep.*, 66, 289, 1982.
472. Leichner, P. K., Klein, J. L., Siegelman, S. S., Ettinger, D. S., and Order, S. E., Dosimetry of 131-I-labelled antiferritin in hepatoma: specific activities in the tumor and liver, *Cancer Treat. Rep.*, 67, 647, 1983.
473. Rostock, R. A., Klein, J. L., Leichner, P., Popher, K. A., and Order, S. E., Selective tumor localization in experimental hepatoma by radiolabeled antiferritin antibody, *Int. J. Radiat. Oncol. Physiol.*, 9, 1345, 1983.
474. Nord, S. and Weissman, I. L., Radiolabeled antitumor antibodies. II. Quantitative analysis of Moloney tumor antigens on Moloney lymphoma cells (LSTRA), *JNCI*, 53, 125, 1974.
475. Greig, W. R., McDougall, I. R., and Halnan, K. E., Treatment with unsealed radioisotopes, *Br. Med. Bull.*, 29, 63, 1973.
476. Bloomer, W. D., McLaughlin, W. H., Neirincky, R, D., and Adelstein, S. J., Astatine-211-tellurium radiocolloid curves experimental malignant ascites, *Science*, 212, 340, 1981.
477. Hawthorne, M. F. and Wiersema, R. J., Preparation of tumor specific boron compounds. I. In vitro studies using boron-labeled antibodies and elemental boron as neutron targets, *J. Med. Chem.*, 15, 449, 1972.

478. Mallinger, A. G., Jozwiak, E. L., Jr., and Carter, J. C., Preparation of boron-containing bovine gamma-globulin as a model compound for a new approach to slow neutron therapy of tumors, *Cancer Res.*, 32, 1947, 1972.

479. Farr, L. E. and Koniwowski, T., Long range effects of neutron capture therapy of cancer in mice, *Int. J. Nucl. Med. Biol.*, 3, 1, 1976.

480. Saigusa, T. and Veno, Y., Calculated responses to a thermal neutron beam for hamster and HeLa cells containing boron-10 at different concentrations, *Phys. Med. & Biol.*, 23, 748, 1978.

481. Dicke, K. A., Jagannath, S., Spitzer, G., Poynton, C., Zander, A., Vellekoop, L., Reading, C. L., Jehn, R. W., and Tindle, S., The role of autologous bone marrow transplantation in various malignancies, *Semin. Hematol.*, 21, 109, 1984.

482. Thomas, E. D., Current status of bone marrow transplantation, *Transplant. Proc.*, 17, 428, 1985.

483. Korbling, M., Hess, A. D., Tutschka, P. J., Kaizer, H., Colvin, M. D., and Santos, G. W., 4-Hydroperoxyclophosphamide, a model for eliminating residual human tumor cells and T-lymphocytes from the bone marrow graft, *Br. J. Haematol.*, 52, 89, 1982.

484. Buckman, R., McIlhinney, R. A. J., Shephard, V., Patel, S., Coombes, R. C., and Neville, A. M., Elimination of carcinoma cells from human bone marrow, *Lancet*, 2, 1428, 1982.

485. Stepan, D. E., Bartholomew, R. M., and LeBien, T. W., In vitro cytodestruction of human leukemic cells using murine monoclonal antibodies and human complement, *Blood*, 63, 1120, 1984.

486. Jansen, J., Falkenberg, J. H. F., Stepan, D. E., and LeBien, T. W., Removal of neoplastic cells from autologous bone marrow grafts with monoclonal antibodies, *Semin. Hematol.*, 21, 164, 1984.

487. Bast, R. C., Jr., De Fabritis, P., Lipton, J., Gelber, R., Maver, C., Nadler, L., Sallan, S., and Ritz, J., Elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement, *Cancer Res.*, 45, 499, 1985.

488. Stong, R. C., Youle, R. D., and Vallera, D. A., Elimination of clonogenic T-leukemia cells from human bone marrow using anti Mr 65,000 protein immunotoxins, *Cancer Res.*, 44, 3000, 1984.

489. Myers, C. D., Thorpe, P. E., Ross, W. C. J., Cumber, A. J., Katz, F. E., Tax, W., and Greaves, M. F., An immunotoxin with therapeutic potential in T cell leukemia: WT1 ricin A, *Blood*, 63, 1178, 1984.

490. Diener, E., Diner, U. E., Sinha, A., Zie, Z., and Vergidis, R., Specific immunosuppression by immunotoxins containing daunomycin, *Science*, 231, 148, 1986.

491. De Fabritis, P., Bregni, M., Lipton, J., Greenberger, J., Nadler, L., Rothstein, L., Korbling, M., Ritz, J., and Bast, R. C., Elimination of clonogenic Burkitt's lymphoma cells from human bone marrow using 4-hydroperoxycyclophosphamide in combination with monoclonal antibodies and complement, *Blood*, 65, 1064, 1985.

492. Uckun, F. M., Ramakrishnan, S., and Houston, L. L., Increased efficiency in selective elimination of leukemia cells by a combination of a stable derivative of cyclophosphamide and a human B-cell-specific immunotoxin containing pokeweek antiviral protein, *Cancer Res.*, 44, 69, 1985.

493. Kaizer, H., Stuart, R. K., Fuller, D. J., Braine, H. G., Serai, R., Colvin, M., Wheram, M. D., and Santos, G. W., Autologous bone marrow transplantation in acute leukemia: progress report on phase I study of 4-hydroperoxycyclophos (4-Hc) incubation of marrow prior to cryopreservation, *Proc. Am. Soc. Clin. Oncol.*, 1, 131, 1982.

494. Gorin, N. C., Douay, L., Laporte, J. P., Lopez, M., Mary, J. Y., Najman, A., Salmon, C., Aegerter, P., Stachowiak, J., David, R., Pene, F., Kantor, G., Deloux, J., Duhamel, E., van den Akker, J., Gerota, J., Parlier, Y., and Duhamel, G., Autologous bone marrow transplantation using marrow incubated with asta Z 7557 in adult acute leukemia, *Blood*, 67, 1367, 1986.

495. Ritz, J., Bast, R. C., Jr., Clavell, L., Hercend, T., Sallan, S. E., Lipton, J. M., Fenney, M., Nathan, D. G., and Schlossman, S. F., Autologous bone marrow transplantation in CALLA positive acute lymphoblastic leukemia after in vitro treatment with J5 monoclonal antibody and complement, *Lancet*, 2, 60, 1982.

496. Ramsay, N., LeBien, T., Nesbit, M., McGlave, P., Weisdorf, D., Kenyon, P., Hurd, D., Goldman, A., Kim, T., and Kersey, J., Autologous bone marrow transplantation for patients with acute lymphoblastic leukemia in second or subsequent remission: results of bone marrow treated with monoclonal antibodies BA-1, BA-2, and BA-3 plus complement, *Blood*, 667, 508, 1985.

497. Reisfeld, T. A., Greene, M. I., and Yachi, A., Monoclonal antibodies — progress in cancer immunobiology and clinical application, *Cancer Res.*, 46, 2193, 1986.

498. Gorin, N. C., Douay, L., Laporte, J. P., Lopez, M., Zittoun, R., Rio, B., David, R., Stachowiak, J., Jansen, J., Cazellas, P., Poncelet, P., Liance, M. C., Voisin, G. A., Salmon, C., Le Blanc, G., Deloux, J., Najman, A., and Duhamel, G., Autologous bone marrow transplantation with marrow decontaminated by immunotoxin T 101 in the treatment of leukemia and lymphoma: first clinical observations, *Cancer Treat. Rep.*, 69, 953, 1985.

499. Bast, R. C., Jr., Ritz, J., Lipton, J. M., Feeney, M., Sallan, S. C., Nathan, D. G., and Schlossman, S. F., Elimination of leukemic cells from human bone marrow using monoclonal antibody and complement, *Cancer Res.*, 43, 1389, 1983.

500. Stong, R. C., Uckin, F., Youle, R. J., Kersey, J. H., and Vallera, D. A., Use of multiple T cell-directed intact ricin immunotoxins for autologous bone marrow transplantation, *Blood*, 66, 627, 1985.
501. Casellas, P., Canat, X., Fauser, A. A., Gros, O., Laurent, G., Poncelet, P., and Jansen, F. K., Optimal elimination of leukemic T cells from human bone marrow with T101-ricin A-chain immunotoxin, *Blood*, 65, 289, 1985.
502. Jansen, F. K., Blythman, H. E., Carriere, D., Casellas, P., Diaz, J., Gros, P., Hennequin, J. R., Paelucci, F., Pau, B., Poncelet, P., Richer, G., Salhi, S. L., Vidal, H., and Voisin, G. A., High specific cytotoxicity of antibody-toxin hybrid molecules (immunotoxins) for target cells, *Immunol. Lett.*, 2, 97, 1980.
503. Bregni, M., De Fabritiis, P., Raso, V., Greenberger, J., Lipton, J., Nadler, L., Rothstein, L., Ritz, J., and Bast, R. C., Jr., Elimination of clonogenic tumor cells from human bone marrow using a combination of monoclonal antibody:ricin A chain conjugates, *Cancer Res.*, 46, 1208, 1986.
504. Uckun, F. M., Ramaskrishnan, S., Haag, D., and Houston, L. L., Heterogeneity in leukemia cell populations: a clear rationale for use of combination protocols for ex vivo marrow purging, *Transplant. Proc.*, 17, 462, 1985.
505. Douay, L., Gorin, N. C., Lopez, M., Casellas, P., Liance, M. C., Jansen, F. K., Voisin, G. A., Baillou, C., Laporte, J. P., Najman, A., and Duhamel, G., Evidence for absence of toxicity of T101 immunotoxin on human hematopoietic progenitor cells prior to bone marrow transplantation, *Cancer Res.*, 45, 438, 1985.
506. Falkenburg, J. H. F., Fibbe, W. E., Veenhof, W. F. J., Koning, F., van Eeden, G., Voogt, P. J., and Jansen, J., Selective removal of clonogenic neoplastic B cells from human bone marrow using anti-HLA-DQ antibodies and complement, *Exp. Hematol.*, 14, 101, 1986.
507. Prentice, H. G., Blacklock, H. A., Janossy, G., Gilmore, M. J. M. L., Price-Jones, L., Tidman, N., Trejdosiewicz, L. K., Skeggs, D. B. K., and Panjwani, D., Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic marrow transplant recipients, *Lancet*, 1, 472, 1985.
508. Waldmann, H., Polliak, A., Hale, G., Cividalli, G., Weshler, Z., Mandor, D., Rachmiiewitz, E. A., Or, R., Weiss, I., Samuel, S., Brautbar, C., and Slavin, S., Elimination of graft-versus-host disease by in-vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1), *Lancet*, 2, 483, 1984.
509. Vallera, D. A., Ash, R. C., Zanjani, E. D., Kersey, J. H., LeBien, T. W., Beverley, P. C. L., Neville, D. M., Jr., and Youle, R. J., Anti-T-cell reagents for human bone marrow transplantation: ricin linked to three monoclonal antibodies, *Science*, 222, 512, 1983.
510. Filipovich, A. H., Vallera, D. A., Youle, R. J., Neville, D. M., Jr., and Kersey, J. H., Ex vivo T cell depletion with immunotoxins in allogeneic bone marrow transplantation: the pilot clinical study for prevention of graft-versus-host disease, *Transplant. Proc.*, 17, 442, 1985.
511. Quinones, R. R., Youle, R. J., Kersey, J. H., Zanjani, F. D., Azemove, S. M., Soderling, C. C. B., LeBien, T. W., Beverley, P. C. L., Neville, D. M., Jr., and Vallera, D. A., Anti-T cell monoclonal antibodies conjugated to ricin as potential reagents for human GVHD prophylaxis: effect on the generation of cytotoxic T cells in both peripheral blood and bone marrow, *J. Immunol.*, 132, 678, 1984.
512. Martin, P. J., Hansen, J. A., Buckner, D. C., Sanders, J. E., Deeg, H. J., Stewart, P., Applebaum, F. R., Clift, R., Fefer, A., Witherspoon, R. P., Kennedy, M. S., Sullivan, K. M., Flournoy, N., Storb, R., and Thomas, E. D., Effects of in vitro depletion of T cells in HLA-identical allogeneic marrow grafts, *Blood*, 66, 664, 1985.
513. Remlinger, K., Martin, P. J., Hansen, J. A., Doney, K. C., Smith, A., Deeg, H. J., Sullivan, K., Storb, R., and Thomas, E. D., Murine monoclonal anti-T cell antibodies for treatment of steroid-resistant acute graft-versus-host disease, *Hum. Immunol.*, 9, 21, 1984.
514. Baldwin, R. W. and Byers, V. S., Monoclonal antibodies in cancer treatment, *Lancet*, 1, 603, 1986.
515. Melino, G., Hazarika, P., Elliott, P., Hobbs, J. R., and Cooke, K. B., Protein-bound daunorubicin as an agent for cancer therapy, *Biochem. Soc. Trans.*, 10, 505, 1982.
516. Melino, G., Elliott, P., Cooke, K. B., Evans, A., and Hobbs, J. R., Allogeneic antibodies (Abs) for drug targetting to human neuroblastoma (Nb), *Proc. Am. Soc. Clin. Oncol.*, 3, 47, 1984.
517. Melino, G., Hobbs, J. R., Radford, M., Cooke, K. B., Evans, A. M., Castello, M. A., and Forrest, D. M., Drug targeting for 7 neuroblastoma patients using human polyclonal antibodies, *Protides Biol. Fluids*, 32, 413, 1985.
518. Melino, G., Drug targeting for neuroblastoma: a case report, *J. Cell. Biochem.*, Suppl. 9A, 445, 1985.
519. Melino, G., Drug targeting for neuroblastoma: a case report, *Protides Biol. Fluids*, 32, 445, 1985.
520. Ghose, T., Guclu, A., Faulkner, J., and Tai, J., Suppression in vivo of the mouse EL4 lymphoma by rabbit antitumor sera, *JNCI*, 58, 693, 1977.

521. Ghose, T., Guclu, A., Tai, J., Mammen, M., and Norvell, S. T., Immunoprophylaxis and immunotherapy of EL4 lymphoma, *Eur. J. Cancer,* 13, 83, 1977.
522. Wright, P. W. and Bernstein, I. D., Serotherapy of malignant disease, *Prog. Exp. Tumor Res.,* 25, 140, 1980.
523. Kirch, M. D. and Hammering, U., Immunotherapy of murine leukemias by monoclonal antibody. I. Effect of passively administered antibody on growth of transplanted tumor cells, *J. Immunol.,* 127, 805, 1981.
524. Young, W. W. and Hakomori, S.-I., Therapy of mouse lymphoma with monoclonal antibodies to glycolipid: selection of low antigenic variants in vivo, *Science,* 211, 487, 1981.
525. Dillman, R. O. and Royston, I., Applications of monoclonal antibodies in cancer therapy, *Br. Med. Bull.,* 40, 240, 1984.
526. Goodman, G. E., Beaumier, P., Hellstrom, I., Fernyhough, B., and Hellstrom, K.-E., Pilot trial of murine monoclonal antibodies in patients with advanced melanoma, *J. Clin. Oncol.,* 3, 340, 1985.
527. Houghton, A. N., Mintzer, D., Gordon-Cardo, C., Welt, S., Fliegel, B., Vadhan, S., Carswell, E., Melamed, M. R., Oettgen, H. F., and Old, L. J., Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma, *Proc. Natl. Acad. Sci. U.S.A.,* 82, 1242, 1985.
528. Rubens, R. D. and Dulbecco, R., Augmentation of cytotoxic drug action by antibodies direct a cell surface, *Nature (London),* 248, 81, 1974.
529. Segerling, M., Ohanian, S. H., and Borsos, T., Effects of metab inhibitors on killing of tumor cells by antibody and comment, *JNCI,* 53, 1411, 1974.
530. Davies, D. A. L. and O'Neill, G. L., In vivo and in vitro effects of tumor specific antibodies with chlorambucil, *Br. J. Cancer,* 28, 285, 1973.
531. Schlager, S. I. and Ohanian, S. H., Correlation between lipid synthesis in tumor cells and their sensitivity to humoral immune attack, *Science,* 197, 773, 1977.
532. Ghose, T. and Cerini, M., Radiosensitization of Ehrlich ascites tumor cells by a specific antibody, *Nature (London),* 222, 993, 1969.
533. Vermund, H. and Gollin, F. F., Mechanisms of action of radiotherapy and chemotherapeutic adjuvants, *Cancer,* 21, 58, 1968.
534. Gill, D. M. and Pappenheimer, A. M., Jr., Structure-activity relationship in diphtheria toxin, *J. Biol. Chem.,* 246, 1492, 1971.
535. Olsnes, S. and Phil, A., Chimeric toxins, *Pharmacol. Ther.,* 15, 355, 1982.
536. Masuho, Y., Kishdia, K., Saito, M., Umemoto, N., and Hara, T., Importance of the antigen-binding valency and the nature of the crosslinking bound in ricin A-chain conjugates with antibody, *Biochem. J.,* 9, 1583, 1982.
537. Schwartz, H. S., Biochemical action and selectivity of intercalating drugs, *Adv. Cancer Chemother.,* 1, 1, 1979.
538. Linford, J. H. and Froese, G., Comparisons of the chemical and biologic properties of triaziquone and triaziquone-protein conjugates, *JNCI,* 60, 307, 1978.
539. Arnon, R., Antibodies and dextran as anti-tumour drug carriers, in *Targeting of Drugs,* Gregoriadis, G. and Trouet, A., Eds., Plenum Press, New York, 1982, 31.
540. Ramakrishnan, S. and Houston, L. L., Immunological and biological stability of immunotoxins in vivo as studies by the clearance of disulfide-linked pokeweed antiviral protein-antibody conjugates from blood, *Cancer Res.,* 45, 2031, 1985.
541. Guclu, A., Ghose, T., Tai, J., and Mammen, M., Binding of chlorambucil with anti-tumor globulins and its effect on drug and antibody activities, *Eur. J. Cancer,* 12, 95, 1976.
542. Carriere, D., Casellas, P., Richer, G., Gros, P., and Jansen, F. K., Endocytosis of antibody ricin A-chain conjugate (immuno-A-toxin) adsorbed on colloidal gold, *Exp. Cell Res.,* 156, 327, 1985.
543. Casellas, P., Bourrie, B. J. P., Gros, P., and Jansen, F. K., Kinetics of cytotoxicity induced by immunotoxins, *J. Biol. Chem.,* 259, 9359, 1984.
544. Ghose, T., Nairn, R. C., and Fothergill, J. E., Uptake of proteins by malignant cells, *Nature (London),* 196, 1108, 1962.
545. Ramakrishnan, S. and Houston, L. L., Comparison of the selective cytotoxic effects of immunotoxins containing ricin A chain or pokeweed antiviral protein and anti-Thy 1.1 monoclonal antibody, *Cancer Res.,* 44, 201, 1984.
546. Blythman, H. E., Casellas, P., Gros, O., Gros, P., Jansen, F. K., Paolucci, F., Pau, B., and Vidal, H., Immunotoxins: hybrid molecules of monoclonal antibodies and toxin subunits specifically kill tumor cells, *Nature (London),* 290, 145, 1981.
547. Sikora, K., Smedley, H., and Thorpe, P., Tumor imaging and drug targeting, *Br. Med. Bull.,* 40, 233, 1984.
548. McCardle, R. J., Harper, P. V., Spar, I. L., Bale, W. F., Andros, G., and Jiminez, F., Studies with iodine-131-labeled antibody to human fibrinogen for diagnosis and therapy of tumors, *J. Nucl. Med.,* 7, 837, 1966.

549. Oi, V. T., Morrison, S. L., Herzenberg, L. A., and Berg, P., Immunoglobulin gene expression in transformed lymphoid cells, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 825, 1983.
550. Ochi, A., Hawley, R. G., Lawley, T., Shulman, M. J., Traunecker, A., Kohler, G., and Hozumi, N., Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 6351, 1983.
551. Deans, R. J., Denis, K. A., Taylor, A., and Wall, R., Expression of an immunoglobulin heavy chain gene transfected into lymphocytes, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 1292, 1984.
552. Boss, M. A., Kenten, J. H., Wood, C. R., and Emtage, J. S., Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in *E. coli, Nucl. Acids Res.*, 12, 3791, 1984.
553. Wood, C. R., Boss, M. A., Kenten, J. H., Calvert, J. E., Roberts, N. A., and Emtage, J. S., The synthesis and in vivo assembly of functional antibodies in yeast, *Nature (London)*, 314, 446, 1985.
554. Carson, D. A. and Freimark, B. D., Human lymphocyte hybridomas and monoclonal antibodies, *Adv. Immunol.*, 38, 275, 1986.
555. Sugden, B., Marsh, K., and Yates, J., A vector that replicates as a plasmid and can be efficiently selected in B-lymphocytes transformed by Epstein-Barr virus, *Mol. Cell Biol.*, 5, 410, 1985.
556. Sorge, J., Wright, D., Erdman, V., and Cutting, A. E., Amphotropic retrovirus vector system for human cell gene transfer, *Mol. Cell Biol.*, 4, 1730, 1984.
557. Williams, D. A., Lemischka, I. R., Nathan, D. G., and Mulligan, R. C., Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse, *Nature (London)*, 310, 476, 1984.
558. Morrison, S. L., Johnson, M. J., Sherzenberg, L. A., and Oi, V. T., Chimeric human antibody molecules: mouse antibinding domains with human constant region domains, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 6851, 1984.
559. Boulianne, G. L., Hozumi, N., and Shulman, M. J., Production of functional chimaeric mouse/human antibody, *Nature (London)*, 312, 643, 1984.
560. Takeda, S., Naito, T., Hama, K., Noma, T., and Honjo, T., Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences, *Nature (London)*, 314, 452, 1985.
561. Neuberger, M. S., Williams, G. T., Mitchell, E. B., Jouhal, S. S., Flanagan, J. G., and Rabbitts, T. H., A hapten-specific chimaeric IgE antibody with human physiological effector function, *Nature (London)*, 314, 268, 1985.
562. Parham, P., Kipps, T. J., Ward, F. E., and Herzenberg, L. A., Isolation of heavy chain class switch variants of a monoclonal anti-DC1 hybridoma cell line: effective conversion of noncytotoxic IgG_1 antibodies to cytotoxic IgG_2 antibodies, *Hum. Immunol.*, 8, 141, 1983.
563. DePhino, R. A., Feldman, L. B., and Scharff, M. D., Tailor-made monoclonal antibodies, *Ann. Int. Med.*, 104, 225, 1986.
564. Rosen, A. and Klein, G., U.V. light-induced immunoglobulin heavy chain class switch in a human lymphoblastoid cell line, *Nature (London)*, 306, 189, 1983.
565. Roder, J. G., Cole, S. P. C., Atlaw, T., Campling, B. G., McGarry, R. C., and Kozbor, D., The Epstein-Barr virus hybridoma technique, in *Human Hybridomas and Monoclonal Antibodies*, Engleman, E. and Foung, S. K. H., Eds., Plenum Press, New York, 1985, 55.
566. Sharon, J., Gefter, M. L., Manser, T., Morrison, S. L., Oi, V. T., and Ptashne, M., Expression of a V_{HC} kappa chrimaeric protein in mouse myeloma cells, *Nature (London)*, 309, 364m, 1984.
567. Neuberger, M. S., Williams, G. T., and Fox, R. O., Recombinant antibodies possessing novel effector functional chimaeric mouse/human antibody, *Nature (London)*, 312, 643, 1984.
568. Van Ness, J., Laemmii, U. K., and Pettijohn, D. E., Immunization in vitro and production of monoclonal antibodies specific to insoluble and weakly immunogenic proteins, *Proc. Natl. Acad. Sci. U.S.A.*, 81, 7897, 1984.
569. Old, L. J., Tumor necrosis factor (TNF), *Science*, 230, 630, 1985.
570. Piper, J. R., Montgomery, J. A., Sirotnak, F. M., and Chello, P. L., Syntheses of x- and y-substituted amides, peptides, and esters of methotrexate and their evaluation as inhibitors of folate metabolism, *J. Med. Chem.*, 25, 182, 1982.
571. Rosowsky, A., Forsch, R. A., Galivan, J., Susten, S. S., and Freisheim, J. H., Regiospecific Y-conjugation of methotrexate to poly(L-lysine). Chemical and biological studies, *Mol. Pharm.*, 27, 141, 1985.
572. Bradwell, A. R., Fairweather, D. S., Dykes, P. W., Keeling, A., Vaughan, A., and Taylor, J., Limiting factors in the localization of tumours with radiolabelled antibodies, *Immunol. Today*, 6, 163, 1985.
573. Linder, R., Alteration of mammalian membranes by the cooperative and antagonistic actions of bacterial proteins, *Biochim. Biophys. Acta*, 779, 423, 1984.
574. FitzGerald, D. J. P., Waldmann, T. A., Willingham, M. C., and Pastan, I., *Pseudomonas* exotoxin-anti-RAC. Cell-specific immunotoxin active against cells expressing the human T cell growth factor receptor, *J. Clin. Invest.*, 74, 966, 1984.

575. Uckun, F. M., Ramakrishnan, S., and Houston, L. L., Increased efficiency in selective elimination of leukemia cells by a combination of a stable derivative of cyclophosphamide and a human B-cell-specific immunotoxin containing pokeweed antiviral protein, *Cancer Res.*, 45, 69, 1985.
576. Fodstad, O. and Pihl, A., Synergistic effect of adriamycin and ricin on L1210 leukemic cells in mice, *Cancer Res.*, 40, 3735, 1980.
577. Sironi, M., Canegrati, M. A., Romano, M., Vecchi, A., and Spreafico, F., Chemotherapy-increased antineoplastic effects of antibody-toxin conjugates, *Cancer Treat. Rep.*, 68, 643, 1984.
578. Raso, V. and Lawrence, J., Carboxylic ionophores enhance the cytotoxic potency of ligand- and antibody-delivered ricin A chain, *J. Exp. Med.*, 160, 1234, 1984.
579. Stauffer, P. R., Cetas, T. C., and Jones, R. C., System for producing localized hyperthermia in tumors through magnetic induction heating of ferromagnetic implants, 3rd Int. Symp.: Cancer Therapy by Hyperthermia, Drugs, and Radiation, *Natl. Cancer Inst. Monogr.*, 61, 4, 1982.